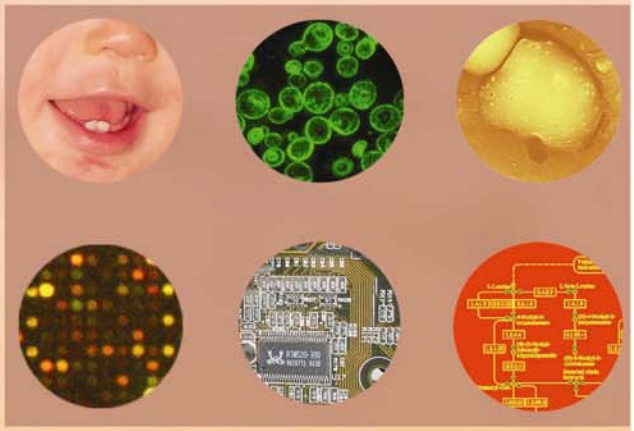

Yeast Genomics on Food Flavours

Sung Ah Schoondermark-Stolk

Yeast Genomics on Food Flavours

YEAST GENOMICS ON FOOD FLAVOURS

Genomics op vorming van smaakstoffen door gist.

(met Nederlandse samenvatting)

ACADEMISCH PROEFSCHRIFT

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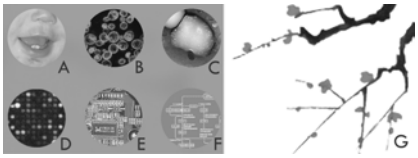

A = taste
B = *Saccharomyces. cerevisiae* (fluorescence microscopy, Bruno Humbel)
C = nucleus of *S. cerevisiae* (scanning electron microscopy, Bruno Humbel)
D = image of cDNA microarray (results described in Chapter 3)
E = computer chip
F = metabolic pathway
G = cherry blossoms

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Savor the Flavor, don't Waste the Taste!

Contents

Chapter one

General introduction

Yeast Genomics on Food Flavours

Since ancient times yeasts have been used for preparing food products. Food fermentation is the oldest 'biotechnology' example we know; brewing of beer, wine, soy sauce, and preparation of bread. Remains of ancient Babylonian and Egyptian civilizations (3000 BC) were discovered, bearing inscriptions and pictures referring to fermented foods [1]. Especially Asia has developed many fermented foods on basis of (vegetable) proteins using fungi in fermentation processes. In Europe and the US, food fermentation has been mainly focussed on food preservation by means of an acid fermentation, whereas properties such as taste, nutritional value and health effects, are more important in Asian fermentations [2]. The actual taste of fermented foods is determined by its odor, taste, colour and texture and can be classed under the general term 'flavour'. The meaning of flavour is according to Merriam-Webster Medical Dictionary: 1a, the quality of something that affects the sense of taste (condiments; give 'flavour' to food) b, the blend of taste and smell sensations evoked by a substance in the mouth (the flavour of ripe fruit) 2, a substance that flavours. In this thesis, the term 'flavour' refers to the second definition.

Many flavour components, responsible for the taste of fermented foods, were identified by techniques like high-pressure liquid chromatography, gas chromatography and mass spectrometry [3, 4]. This identification intensified the further development of the flavour industry. Although food consists of a mixture of many different chemical substances, the aroma of fermented foods is determined by more or less volatile compounds of a few chemical classes: aldehydes, ketones, organic acids, esters, alcohols, sulfur compounds, heterocyclic compounds like lactones and furanones, pyrazines, fenols and terpenes. Among these classes, the branched chain alcohols, 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol, collectively called fusel alcohols, were present as high impact compounds, responsible for the specific taste of fermented foods [5], table 1.

The importance of yeast in fermentation was discovered by Louis Pasteur in the 19th century [6]. In 1888, specific yeast strains, involved in brewing, were isolated [7] and are now all included in the species *Saccharomyces cerevisiae* [28].

Table 1. Typical composition of fusel alcohols, derived from food fermentation (vol% of the total flavour compounds)

Component	Vol (%)
2-Methyl-1-butanol	43.6
3-Methyl-1-butanol	38.7
Isobutanol	12.2
Propanol	1.20
Trimethylpyrazine	0.24
Butanol	0.21
Ethyldecanoate	0.16
2,5-Dimethylpyrimidine	0.12
3-Ethyl-2,5-dimethylpyrazine	0.12
Ethyldodecanoate	0.08
Ethyloctanoate	0.06
2-Ethyl-3,5-dimethylpyrazine	0.05
2-Ethoxy-ethylacetate	0.05
3-Methylbutylacetate	0.04

Since then, *S. cerevisiae* has become a model eukaryotic organism for scientific research. Besides *S. cerevisiae,* the salt-tolerant yeast *Zygosaccharomyces rouxii* was identified as an important aroma producer during the soy sauce fermentation [9]. In the traditional soy sauce fermentations, said to be started by a priest from China in about 1250 BC, soybeans and wheat were mixed together with water and salt (koji) and were left for six months. During that time, the starch and proteins of the soybeans and wheat were hydrolysed, fermented and converted into various sugars, amino acids, fusel alcohols and organic acids [29].

Nowadays many synthetic compounds, derived from the petrochemistry or other natural chemicals, are added in the food preparation process for flavouring purposes. Food legislation classifies those flavours as 'artificial'. Flavours that are generated during food processing, heating or fermentation, are considered as 'natural' [30]. These regulations, together with the consumer demands for healthy food, minimally processed food, and 'clean' labelled food, caused a renewed interest in flavour formation, derived from the metabolisms of living cells.

Production of fusel alcohols

It is known that flavour substances formed by yeast, *S. cerevisiae* and the soy yeast *Z. rouxii*, give the characteristic flavour to fermented foods [10, 11]. Nevertheless, the metabolic pathways, leading to fusel alcohol production remain obscure, which makes the fermentation process difficult to control.

The formation of fusel alcohols is closely related to branched chain amino acid (BCAA) metabolism. It is supposed that fusel alcohols are formed from branched chain amino acids via a route, called the Ehrlich pathway [12]. Leucine, iso-leucine and valine are converted into fusel alcohols in three steps (Fig. 1). First, the amino groups are removed by transamination, catalysed by a branched chain amino acid transferases. Two genes have been identified, *BAT1* and *BAT2,* encoding enzymes responsible for the first conversion step [13]. The remaining oxo-acids are decarboxylated by a decarboxylase. This reaction is possibly performed by pyruvate decarboxylases (PDCs). However, PDCs are not rate limiting for fusel alcohol formation [14]. It has been proposed that *YDL080c* and *ARO10*, PDC-like enzymes, play important roles in 2-oxoglutarate decarboxylation [15]. The third step is the reduction step of the aldehyde products to branched chain alcohols, catalysed by an NAD(P)H-dependent alcohol dehydrogenase (ADH). Candidates for this reaction are Adh and putative Adh-coding genes [16]. Within these genes, two NADPH-dependent Adhs (*ADH6, ADH7*) are identified as main alcohol dehydrogenases, involved in the formation of fusel alcohols [17, 18].

Via the Ehrlich pathway leucine, iso-leucine and valine are converted into respectively 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-butanol (active amyl alcohol) and 2-methyl-1-propanol (isobutyl alcohol) [12, 15, 19]. Before the branched chain amino acids can enter the Ehrlich pathway, they have to enter the cell. The yeast cell possesses several branched chain amino acid permeases (BAPs) to transport the BCAAs into the cell: Gap1, Bap2 and Bap3 [20]. It is supposed that the formed fusel alcohols do not need transporters and diffuse freely out of the cell.

Genetic studies, performed in order to enhance fusel alcohol production by yeast, were so far unsuccessful. It was observed that neither the amino acid permeases [20], the amino acid transferases [21] or the decarboxylases [14] were rate-limiting steps in the fusel alcohol production. Overexpression of these genes did not enhance fusel alcohol formation.

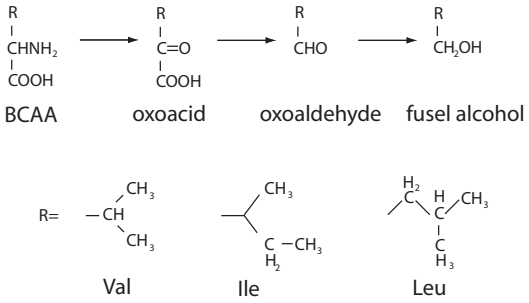

Figure 1. Ehrlich Pathway. Simplified representation of the catabolism of BCAAs. Side groups are indicated for valine, iso-leucine and leucine

Genomic analysis

The great advantage of using *S. cerevisiae* above other micro-organisms in research, is the tremendous amounts of information available for this yeast. In 1996 the whole genome was sequenced and made assessible to the scientific community. The *S. cerevisiae* genome is formed by 16 chromosomes, carrying more than 6000 so-called open reading frames (ORFs), potentially encoding proteins [8].

One of the most powerful new technologies, evolved after the announcement of genetic code of *S. cerevisiae,* was the development of cDNA microarrays. While a number of techniques had been used to estimate the relative abundance of mRNA for specific genes [23], with cDNA microarrays it became possible to investigate changes of mRNA levels of all ORFs in one experiment. The concept of microarrays started in 1975 with the introduction of the Southern blot [24]. The first 'modern' microarray analysis was introduced in 1995, with 45 oligonucleo-tide sequences present on one array [25]. The use of cDNA microarray system requires the following basic steps for investigating gene expression of two related cell types or conditions: 1) isolation and subsequently quality control of mRNA derived from the cells, 2) preparation of labelled cDNA from the mRNA by reverse transcription in the presence of labelled dUTP, 3) hybridization of the labelled cDNAs to the ORFs printed on a matrix, the cDNA microarray, 4) quantitative analysis from the degree of hybridization, which represent the relative abundance of the mRNAs and 5) data analysis or so-called datamining (Fig. 2).

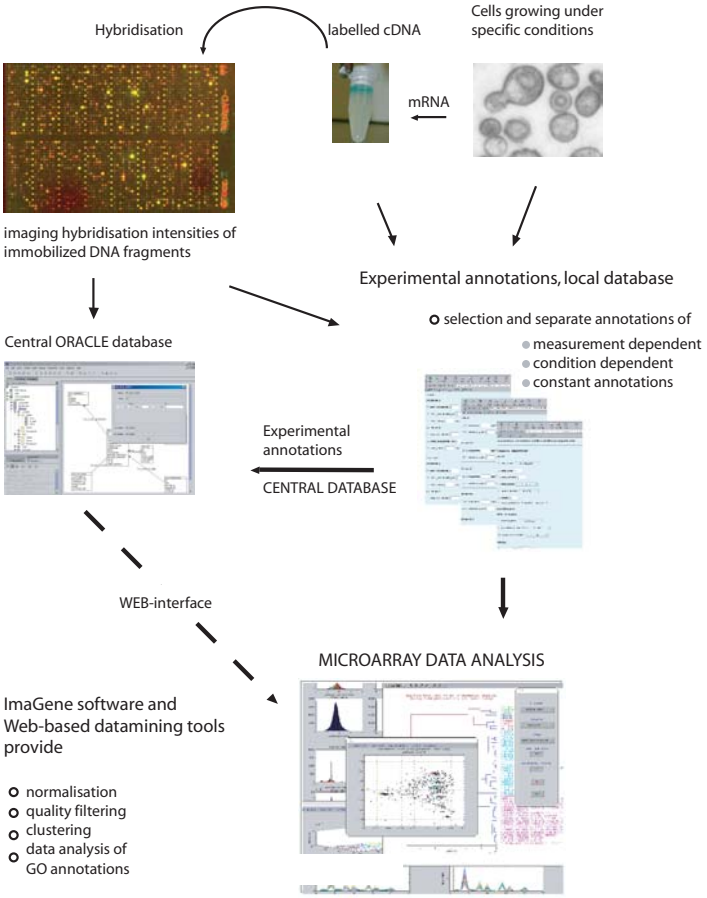

Figure 2. The use of cDNA microarrays. Yeast cells were harvested under different fermentation conditions. Information about the yeast background and fermentation conditions are stored in a central Oracle-based database. A filter array was probed with ^{33}P-labeled cDNA derived mRNA, isolated from harvested cells, and the signal intensity of each ORF was quantified by phosphorimaging. After imaging, spot intensities are determined by ImaGene software and converted in txt-files. Image files and txt-files are also stored in the central database. Datamining is performed by using MS Excel 2000, in combination with SAM, Cluster and Treeview and GO TermFinder.

In 1997 of the first genome-wide gene expression analysis of *S. cerevisiae* was performed with a cDNA microarray system, containing the entire 6,400 ORFs (open reading frames) of the yeast *S. cerevisiae* [26]. cDNA microarray analyses showed that numerous genes and pathways in yeast responded to environmental changes such as the depletion of glucose. By looking for upstream regulatory elements and transcription, 217 factors were identified in genes of particular clusters. These experiments revealed the power of the microarrays.

It is clear that the development of array-based technologies in these past 8 years caused a fundamental shift in the study of living organisms. Compared to the numerous efforts to elucidate the pathways for fusel alcohol production, looking genome-wide at the expression patterns of cells provide a solution to the problem of which specific genes to select for study. Since all ORFs are present on a single array, each microarray experiment accomplishes the equivalent of thousands of genetic tests in parallel. The application of DNA microarrays and identification of possible genes involved in the pathway of fusel alcohol formation, has been the main object of study in this thesis (Fig. 3).

Outline of the thesis

During food fermentation processes, such as Japanese soy sauce fermentation, yeast is responsible for the characteristic aroma profile. Many studies showed that extracellular conditions during food fermentation had a great impact on the final food flavour [27]. For example, the level of fusel alcohols in fermented foods is influenced by temperature, oxygen, NaCl-levels and available nitrogen sources. These extracellular changes affecting yeasts' metabolic routes, thereby influencing the production of the fusel alcohols.

The aim of the research for this thesis was to elucidate genes and routes, leading to fusel alcohol formation by cDNA microarrays, in order to provide targets for directed mutagenesis which will ultimately lead to strains with a more efficient flavour production. The project covered new approaches for systematic analyses of gene function, including comparative genomics, expression profiling, bioinformatic studies and metabolite profiling. When we started our studies, little was known about the genome of *Z. rouxii*, generating a major problem in the elucidation of the underlying metabolism towards fusel alcohols.

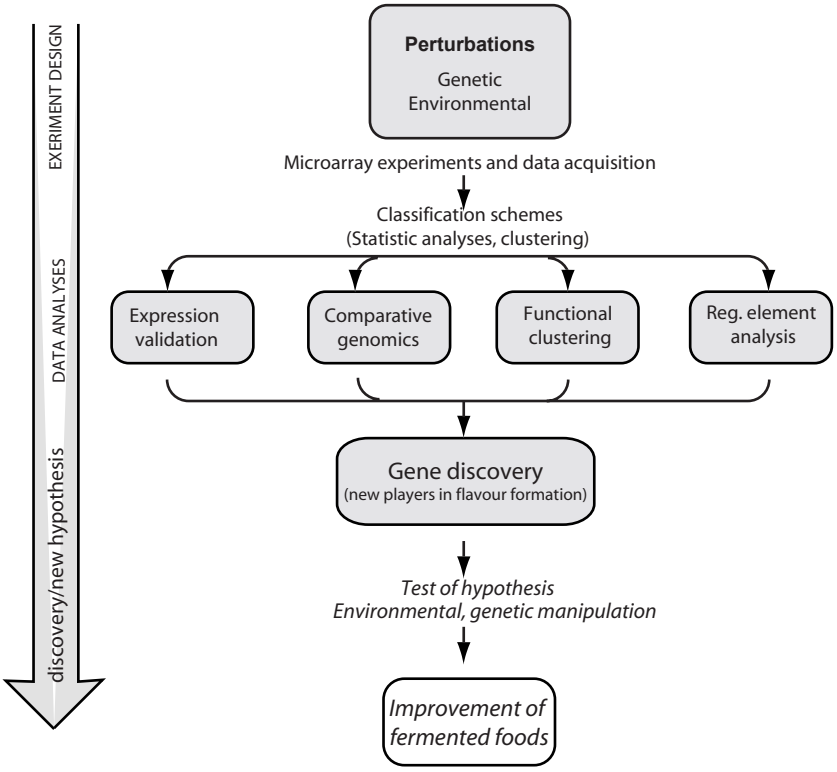

Figure 3. Schematic representation of the use DNA microarrays in this thesis. Microarray experiments are designed to measure the expression patterns of genes in a specific set of conditions. After data acquisition, gene expression changes are validated. The microarrays are also used to identify genes across species (chapter 2) and to identify genes, mapped to biological pathways (chapter 3, 4, 5). In chapter 3 and 4, microarray data are used for regulatory element analysis. Standardization of data formatting, annotation, normalisation and clustering, as well as new databases to store and mine gene expression information, will facilitate information extraction from the microarray datasets. Future experiments by conventional approaches are needed to ultimately proof the hypothesis built on the microarray experiments.

In chapter 2, we demonstrate gene expression profiling for *Z. rouxii* with commercially available *S. cerevisiae* genefilters. In this chapter, an overview is given of all *Z. rouxii* genes and salt stress responsive genes, detected by cross hybridization of cDNA from *Z. rouxii* genes with *S. cerevisiae* filters. At the same time, it was determined that the genome of *Z. rouxii* consisted of seven chromosomes, 12.8 Mb in total. Gene order comparison revealed that gene organization was partially conserved in both species [9].

Available information on routes leading to fusel alcohol formation in *Z. rouxii* and *S. cerevisiae* showed that fusel alcohols are most likely to be derived from branched chain amino acids (BCAAs). Features of expression profiles of flavour producing yeasts to elucidate underlying pathways are further investigated in *S. cerevisiae* and described in chapter 3-5. A few steps involved in the degradation of BCAAs have been known for a long time, including a transaminase step leading to the accumulation of oxoacids. The two enzymes, catalyzing this reaction have been discovered some time ago. Bat1p and bat2p are the two clearly defined proteins involved in degradation of BCAAs. Although several studies indicate the involvement of these enzymes in fusel alcohol production, it was not clear which effects these enzymes had on the final flavour formation. Since most yeast fermentations start with glucose consumption in excess of glucose with fermentative growth, they eventually end up with ethanol consumption. We set up gene deletion studies to assess the influence and importance of these enzymes in fusel alcohol production. The impact of BCAATs on flavour formation under fermentative growth and respiratory growth conditions was investigated and is described in chapter 3.

This led to the discovery that expression of *BAT2* was essential for fusel alcohol production during ethanol consumption. No 3-methyl-1-butanol was produced by the *bat2Δ* - and *bat1Δ bat2Δ*-deletion strains. Although there are many steps between gene expression and final metabolite formation, cDNA microarray data revealed consistent expression profiles of *BAT1* and *BAT2,* with the final measured fusel alcohol concentrations. The gene expression profiles on the cDNA microarrays showed that *BAT1* was strongly expressed in glucose grown cells, while a strong *BAT2* expression was found in ethanol grown cells. This was in agreement with the unaffected fusel alcohol production in cells grown on glucose, which lack the *BAT2* gene and the severe effect on the fusel alcohol production by deletion of *BAT2*. In addition, the transcription level of several genes coding for a transaminase was affected when one of both of the BCAATs were deleted. However, when *BAT2* was deleted in ethanol grown cells, no

significant enhanced gene expression of other transaminases was observed. Furthermore gene cluster analyses revealed that *BAT2* was co-expressed with several genes containing one of more stress responsive elements (STREs) and that *BAT2* itself possessed 2 STREs.

We also have been able to demonstrate that fusel alcohol accumulation was accompanied by the presence of other aroma metabolites. The metabolite formation and gene expression profiles going with 3-methyl-1-butanol production is described in chapter 4. Genome-gene expression analyses provided clues on pathways leading to flavour formation and fusel alcohol metabolism.

Chapter 5 describes the combination of high throughput metabolic profiling with the information of from genome-wide transcription data. In order to establish under which conditions transcription profiling was optimal, several metabolic profiling arrays of 96 different fermentation conditions were screened. An optimal fermentation condition for 3-methyl-1-butanol production was identified at pH 3.0 and 0% NaCl. A subset of 747 genes was significantly induced or repressed when compared to standard cultivation conditions with pH, identified as optimal condition. Further analyses revealed an interesting subset of 7 genes, encoding for enzymes related to the 3-methyl-1-butanol pathway, in addition 4 genes involved in NAD-metabolism, and 5 genes involved in the TCA-cycle and glutamate metabolism were identified as targets for the improvement of fusel alcohol production. Based upon the fact that a few specific genes could be extracted from genome wide data analysis, we concluded that method of high throughput metabolite screening and cDNA microarray analyses is a rapid and valuable approach for identification of interesting target genes, for example for the use in industrial processes.

Chapter 6 presents the development of microarrays and microarray analyses in retrospective in the context of our project. Special attention is being paid to the rapid development of array technology during the course of the project.

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30. Food legislation http://www.fda.gov/

CHAPTER TWO

Identification of salt-induced genes of *Zygosaccharomyces rouxii* by using *Saccharomyces cerevisiae* GeneFilters®

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Abstract

Yeast GeneFilters® containing all *Saccharomyces cerevisiae* ORF sequences, elucidated gene activity in osmotolerant yeast *Zygosaccharomyces rouxii*. Labelled cDNA derived from *Z. rouxii* cells were targeted to spotted *S. cerevisiae* ORFs. Approximately 90-100% homology of *Z. rouxii* genes with those of *S. cerevisiae* was required for definitive identification of the cDNAs hybridized to GeneFilter®. Hybridized labelled cDNAs were visualised as small spots on the microarray, providing simultaneous information of homologous genes present in *Z. rouxii* and of their level of gene activity. Cross-hybridization of the GeneFilters® displayed 155 as yet unidentified genes of *Z. rouxii* hybridizing to *S. cerevisiae* ORFs. From those 155 genes, the activity of 86 genes was influenced as a result of NaCl-stress. Twenty four percent of *Z. rouxii* genes revealed a different transcription behaviour following NaCl-stress in comparison with *S. cerevisiae*. All of these genes had no previously defined function in salt metabolism in *Z. rouxii*. Therefore, crosshybridization of GeneFilters® proves to be an appropriate and straightforward method for screening transcripts in *Z. rouxii,* which provides an extension of the knowledge of genes present in a yeast genus other than *S. cerevisiae*.

Introduction

Zygosaccharomyces rouxii, a typical salt-tolerant yeast, is an important aroma producer in Japanese type soy sauce fermentations [1]. This type of fermentation takes place in a high-salt environment, where moulded soybeans and wheat are mixed with a brine solution of 17% (w/v) salt [2]. The yeast responds to this hyperosmotic stress by accumulating compatible solutes, mainly glycerol [3, 4]. The induction of compatible solutes may be mediated by a mitogen-activated protein (MAP) kinase cascade, which is called the high osmolarity glycerol (HOG) response pathway. The soy yeast *Z. rouxii* possesses at least one putative MAP kinase gene (*ZrHOG).* The *ZrHOG* gene was discovered due to its homology to the *Saccharomyces cerevisiae HOG1* [5, 6]. Although it is questioned whether glycerol synthesis may occur via the HOG-pathway, two putative glycerol-3-phosphate dehydrogenases, ZrGpd1p and ZrGpd2p, both 73% identical to *S. cerevisiae* Gpd1p, have been identified in *Z. rouxii* [7]. Two isogenes of the *S. cerevisiae* halometric responder *GCY1, ZrGCY1* and *ZrGCY2*, were also cloned from a *Z. rouxii* strain. The genes coding for glycerol dehydrogenases, ZrGcy1p and ZrGcy2p, showed 62% homology to *S. cerevisiae* Gcy1p [7].

Many components, underlying signalling pathways that control biochemical reactions for salt stress protection, have been more extensively investigated in *S. cerevisiae*. Since the availability of the complete genome of *S. cerevisiae,* technologies to analyse genome-wide transcript patterns of this yeast have been rapidly developed and applied [8]. Several microarrays for genome-wide expression analysis have been performed in order to study osmostress [9-13]. The microarray data sets demonstrate that a much larger fraction of the genome is involved in the response to environmental changes than previously assumed. New sets of induced and repressed genes were identified from many different functional families. It has been reported that genes involved in nucleotide and amino acid metabolism responded in the earliest phase of salinity stress, followed by ORFs related to intracellular transport, protein synthesis and translocation [13]. Various transcripts related to energy production, respiration, detoxification, the major facilitator superfamily, metabolism of energy reserves, nitrogen and sulfur compounds, lipid, fatty acid/isoprenoid biosynthesis and a substantial number of previously uncharacterised genes, were also found to be upregulated upon salinity stress.

The similarity between *Z. rouxii* and *S. cerevisiae* towards the salinity stress response and the existence of several homologous genes involved in salt tolerance raises the question about the possibility to identify salt-induced genes in *Z. rouxii,* using commercially available *S. cerevisiae* microarrays. *Z. rouxii* was recently classified as closely related to *S. cerevisiae*, based on conservations of gene order and gene orientation [14, 15]. Given the fact that *Z. rouxii* genes identified so far include functionally and structurally closely related *S. cerevisiae* counterparts, a search was conducted for *Z. rouxii* genes regulated by salinity stress by the use of GeneFilters®, containing the protein-coding sequences of the *S. cerevisiae* genome. Using this approach, various genes active in a high-salt environment such as soy sauce fermentation, could be identified in a rapid and straightforward manner.

Materials and Methods

Yeast Strains and Growth Conditions

For wild type yeast, *Saccharomyces cerevisiae,* strain CEN.PK113-7D was used. The wild type *Zygosaccharomyces rouxii* strain used in this study, was the Chinese *Z. rouxii* strain AS 2.1521. Cells were grown at 30 °C on non-selectivity YPD medium (1% yeast extract, 2% peptone, 2% glucose) to an A_{600} of 1.0 (± 0.2). At this point cells were collected to determine expression profiles under zero stress. Osmotic stress was applied by transferring the cells to fresh YPD medium containing NaCl. *S. cerevisiae* was transferred to 0.7 M NaCl. Samples were taken after 45 minutes salinity stress, when mRNA levels of representative osmoresponsive genes in *S. cerevisiae* were maximal [16]. In order to compare the transcriptional behaviour of *Z. rouxii* with that of *S. cerevisiae, Z. rouxii* cells were transferred to a medium containing 1.4M NaCl. This NaCl-concentration was chosen to achieve a similar salinity response, since for both yeasts this was approximately 40% of the maximum salt concentration they were able to survive in [17]. Samples of both yeasts were taken 45 minutes following salinity stress, in order to maintain identical conditions. Samples were immediately snap frozen in liquid nitrogen after collection and stored at –80 °C.

Cell Extract Preparation

Samples were thawed on ice and the cells were lysed by shaking on glass beads in phenol and 1% SDS in mRNA extraction buffer (100 mM Tris-HCl [pH 7.5],

100 mM LiCl, 1 mM EDTA, 10 mM iodioacetate) at 3200 rpm in a Mini-BeadBeater-8™ (BioSpec Products, Inc.). *S. cerevisiae* cells were lysed in 1 cycle of 90 seconds. *Z. rouxii* cells were lysed for three times 90 s cycles in the Mini-BeadBeater-8™, with 30 s intervals on ice. Cell lysates were separated from glass beads and cell debris by centrifugation at 14000 rpm at 4 °C for 10 minutes. The aqueous phase was extracted by phenol followed by phenol/chloroform/ isoamyl alcohol and chloroform extractions. Total RNA was precipitated by 40% potassium acetate pH 5.6 in ethanol. RNA was resuspended in DEPC-treated water and their OD_{260} values were measured to calculate their total RNA concentrations. The quality of the isolated RNA was also checked on a 1% agarose gel.

Synthesis of [33P]CTP-labeled cDNA

4 μg of mRNA and 2 μg of oligo(dT) (10-20-mer mixture, Life Technologies, Inc.) were mixed in 4 μl of water, heated for 10 min at 70 °C, and then chilled on ice. The following components were added: first strand buffer (Life Technologies, Inc.), 0.1 M dithiothreitol (Life Technologies, Inc.), 1 mM dATP, dGTP, and dTTP, 300 U Superscript II reverse transcriptase (Life Technologies, Inc.), and 100 μCi [^{33}P]CTP (Amersham). The mixture with a total volume of 30 μl was incubated at 37 °C for 90 min. The cDNA probe was then purified by passage through a Sephadex G-50 column.

GeneFilter® hybridization

GeneFilter® membranes (Life Technologies, Inc) were washed for 5 min in boiling 0.5% SDS. The membranes were prehybridized for 4 h with 5 ml MicroHyb solution (Life Technologies, Inc) at 42 °C in a roller oven (Thermo Hybaid). The purified cDNA probe was denatured for 3 min at 90 °C and added to the prehybridization mixture. After overnight hybridization at 42 °C, filters were rinsed twice with 2 × SSC and 0.1% SDS and incubated at 50 °C for 20 min. Filters were then transferred to a plastic box, and washed with 0.5 × SSC and 0.1% SDS at room temperature for 15min. The GeneFilters® gave an unreliable image result and showed a non-specific binding affinity when the hybridization temperature, washing temperature, or a combination of those, was lowered. A phosphorimager SI (Molecular Dynamics) was used to obtain a digital image of the filters after an exposure time to the phosphor imager screen

for 120 hours. To diminish quantification and reproducibility problems, all filters used originated from the same batch. Filters were used only once since the quality of filters seriously declined after stripping.

Data analysis and spot validation

Images were scanned at 50 micron resolution in ImageQuant and were imported into the ImaGene® 4.0 microarray analysis software (BioDiscovery). ImaGene® 4.0 was used for a preliminary quantification of spot intensities. Each appearing genespot was averaged and validated. A threshold value of 1 was given to negative or empty spots. Spots, which possessed the threshold value of 1, with and without NaCl-stress, were excluded from further processing. Inferior quality or poorly hybridized spots were also excluded from further processing. To identify poor quality spots, the standard deviation of the spot signal was divided by the spot intensity mean. When the calculated value exceeded the threshold of 0.5 ($\sigma_s \cdot M_s^{-1}$> threshold), the spot was marked as a poor quality spot and was left out of consideration.

To determine the induction or repression of gene activity, all spot intensities were normalized against total spot intensity. The relative mRNA levels determined in this way gave an indication whether a gene was strongly or poorly expressed. To compare the microarray data of *S. cerevisiae* with previous results [9], spots were also normalized against *IPP1,* a cytoplasmic inorganic phosphatase, which is not affected by osmostress [16]. No difference in normalization against all spots or normalization against *IPP1* was obtained. In order to avoid normalization errors for the cross-hybridized filters, spots were also normalized against *YGR058W*. The ORF *YGR058W* with an unknown function was chosen, because it was clearly visible on the filters, not influenced by salt stress and exhibited an average spot value. Fig 1 shows a part of the filter, hybridized with *S. cerevisiae*- or *Z. rouxii*-labelled cDNA, on which *YGR058w* is present. No discrepancies were obtained by normalization against all spots or against *YGR058w.*

A total of 8 DNA microarray experiments were performed, including duplicate experiments. 4 microarrays were used to detect gene expression in either *S. cerevisiae* or *Z. rouxii* when no NaCl was added. 4 microarrays were used to detect gene expression in either *S. cerevisiae* or *Z. rouxii* when salinity stress was applied.

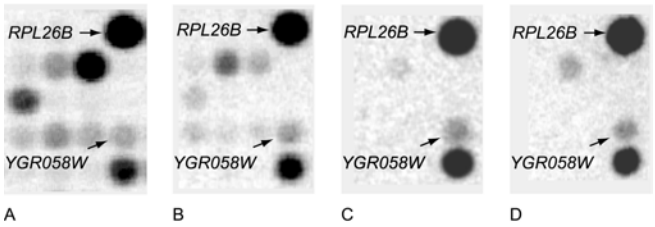

Figure 1A-D. Part of an image scan of a GeneFilter® hybridized with ^{33}p cDNA. A). *Saccharomyces cerevisiae* no NaCl-stress. B). *Saccharomyces cerevisiae* NaCl-stressed cells. C). *Zygosaccharomyces rouxii* no NaCl-stress. D). *Zygosaccharo-myces rouxii* NaCl-stressed cells. *YGR058W* as shown on this scan, was not influenced by the presence of NaCl. This ORF, coding for an unknown gene, is used for normalization. Gene coding for ribosomal protein L26 (*RPL26B*), also shown on this filter part, is one of the most intense spot present on cross-hybridized filters.

Genes whose mRNA level after addition of NaCl was at least 3.0-fold higher or lower than before the osmotic shock were considered as genes influenced by salinity stress.

Results and discussion

GeneFilter® cross-hybridization

For each microarray experiment *S. cerevisiae* or *Z. rouxii* cells were grown on YPD. When the cultures reached A_{600} 0.8-1.2 they were subjected for 45 minutes to either fresh YPD containing no NaCl or YPD containing 0.7 M NaCl or 1.4 M NaCl, respectively. Labelled "target" cDNA was prepared from cultures and hybridized to the GeneFilters® as described in Materials and Methods. Filters were analysed by ImageQuant®. Fig 2 shows an image scan of a hybridized microarray.

For GeneFilters® hybridized with *S. cerevisiae* labelled cDNA, expression values were normalized against *IPP1,* a gene coding for a cytoplasmic inorganic pyrophosphatase and *YGR058W,* an ORF with an unknown function. *IPP1* was chosen because its expression profile is not influenced by osmotic stress [16], *YGR058W* was also not influenced by the presence of NaCl and unlike *IPP1* it appeared also on the cross-hybridized filters (Fig. 1). Furthermore, NaCl did not

influence the total amount of transcript in the cell. No differences in expression values were obtained when normalized against *IPP1, YGR058W* or against all spots. The overall values for the homologous hybridization were in agreement with previously published Northern blot [16] and microarray data sets [9, 18]. Data from microarray and Northern analyses showed that microarray values were reproducible and reliable representatives of gene transcription levels in the cell.

In addition to the complications experienced when interpreting spot intensities as gene expression, we encountered further difficulties due to the cross-hybridization. Spot intensity values obtained by hybridization of *Z. rouxii* labelled cDNA to *S. cerevisiae* ORFs on the filters were approximately 2 - 8 times lower compared with hybridization values obtained using labelled *S. cerevisiae* cDNA. Here, the spot intensities are not only dependent on the amount and length of the cDNA used for hybridization, but in our case also on the homology between *Z. rouxii* and *S. cerevisiae*. Therefore, it was difficult to compare *Z. rouxii* spot values directly with those of *S. cerevisiae*.

Cross-hybridization of *Z. rouxii* labelled cDNA with *S. cerevisiae* GeneFilter® exhibited a small number of hybridizing spots. A total of 155 ORFs of the *Z. rouxii* genome was reproducibly detected by this approach (displayed in table 1 and 2). Stringent hybridization and washing conditions were sustained as in homologous hybridization to maintain reliable spot hybridization and to keep a low filter background signal (Fig. 2).

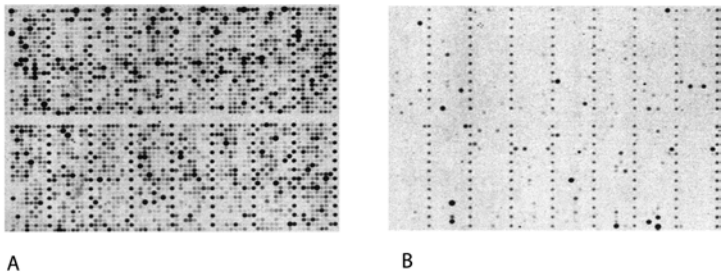

Figure 2A-B. A). An image scan of a *S. cerevisiae* GeneFilter® hybridized with *S. cerevisiae* ^{33}p cDNA. B). An image scan of a *S. cerevisiae* GeneFilter® hybridized with *Z. rouxii* ^{33}p cDNA. 3072 protein-coding sequences of the *S. cerevisiae* genome are present on one filter.

Monitoring active genes during salinity stress

Z. rouxii transcripts of non-stressed cells, which significantly hybridized to spotted *S. cerevisiae* ORFs, differed in hybridization pattern from that of salinity stressed cells. Expression ratios were calculated by dividing mean spot intensities from cells with a 1.4 M NaCl-shock, by spot intensities from cells grown without NaCl. Spot intensities were normalized against total spot intensities. To ensure that the ratios obtained for up regulation or down regulation were not caused by normalization errors, spot intensities were also normalized against *YGR058W,* as described above (Fig. 1). Genes with larger than 3-fold increase in intensity following salt stress, were considered as being positively upregulated. On the other hand, genes, in which the intensity dropped by over 3-fold, were considered as repressed upon salt shock. Using these criteria, 86 *Z. rouxii* genes were found to respond to the osmostress. The expression of 19 genes increased, whereas the expression of 67 genes was decreased. 69 genes were not significantly changed in their expression in the presence or absence of NaCl. Out of 155 genes, 67% showed similar gene expression ratios compared with *S. cerevisiae*. Table 1A lists up or down regulated genes that showed similarity in both yeasts. A list of hybridized genes, which were not significantly changed in expression, is presented in table 1B.

The strongest spot intensities on the cross-hybridized GeneFilter® were observed for the gene *RPL26B* coding for ribosomal protein L26 and three putative ORFs (*YGR069W, YKR040C* and *YNL143C*). The genes were also strongly expressed in *S. cerevisiae*. *RPL26B* is displayed on figure 1, together with *YGR058W*. DNA microarray mRNA ratios of these genes were not significantly influenced upon salinity stress. None of the putative ORFs have a known role or function in the yeast cells and no related proteins have been sequenced in organisms other than *S. cerevisiae*.

Table 1A. List of *S. cerevisiae* genes, which have *Z. rouxii* homologous that show similar up or down regulation following NaCl-stress. Similarities in expression trends induced by salinity stress the different yeast strains. Gene expression of *Z. rouxii* and *S. cerevisiae* was influenced by a salt shock of 1.4 M and 0.7 M, respectively. Expression ratios were scored as "down" or "up" when the genes showed a 3-fold de- or increase in expression. Genes are ordered by their cellular function according to the MIPS Yeast Functional categories [22]. Some genes are present in more than one category.

Ratio Trend	Gene Function	Gene name
Down	Biogenesis	*UTR2*
	Cell Growth, Cell Division and DNA synthesis	*MCM3, POP2, NDD1, BMH2, RIM4, RAD16, YRF1-1*
	Cellular Organisation	*ANP1, AZF1, ENT1, ENT2, HAP2, HSL7, IXR1, LYS2, MCM1, MCM3, MED2, NAB3, NOP1, PAB1, PCF11, PDR1, PET112, PHO2, POP2, QCR7, QCR9, RAD16, RLM1, RSC1, RPS11B, SNF5, TBS1, UBR1, YAP1801*
	Energy	*QCR7, QCR9*
	Metabolism	*GRR1, LYS2, MCM1, PHO2, POP2, SNF5*
	Protein Synthesis	*ANP1, FYV9, PAB1, PET112, RPS11B, UBR1, YAP1801*
	Rescue, Defence, Apoptosis	*GRR1, MCM1, PDR1, RAD16, YGL128C, ZDS1*
	Signal Transduction	*BMH1, BMH2, RLM1*
	Transcription	*AZF1, HAP2, HIR3, IXR1, MCM1, MED2, NAB3, NOP1, PAB1, PCF11, PDR1, PHO2, POP2, PSP2, RLM1, RSC1, SNF5, SWR1, TAF61, TBS1, YIL105C, YIL130W, ZMS1*
	Transport mechanism	*ENT1, ENT2, GRR1, YAP1801*
	Protein of unknown function	*CBK1, PSP1, VID31, YBL081W, YBL109W, YBR113W, YDR154C, YDR291W, YDR544C, YGR073C, YGR086C, YGR237C, YJR041C, YJR115W, YKR075C, YML053C, YMR124W, YNL338W, YOR267C, YPR022C,*
Up	Protein of unknown function	*YNL041C, YOR225W*

Table 1B. List of active *S. cerevisiae* genes, which have *Z. rouxii* homologous that show no significant change in gene-expression following NaCl-stress. Expression ratios were scored as "unchanged" when the genes displayed less than 3-fold difference in gene expression after application of NaCl-stress. Consequently, the clearly visible spots were considered to be expressed at a constant level and not affected by the presence of NaCl. Genes are ordered by their cellular function according to the MIPS Yeast Functional categories [22]. Some genes are present in more than one category.

Ratio Trend	Gene Function	Gene name
Unchanged	Biogenesis	*CBF5, CGR1*
	Cell Growth, Cell Division and DNA synthesis	*BFR2, RLF2, ROM1, SIM1*
	Cellular Organisation	*CBF5, CBP3, DBP2, DRS1, NMD2, NOP5, PEX8, QCR6, RLF2, RPL26B, SAS10, SIK1, SEC16, SNF1*
	Chromatin Chromosome structure	*MCD1*
	Differentiation	*YEL033W*
	Energy	*QCR6*
	Metabolism	*NMD2, SNF1, YBR030W*
	Protein Synthesis	*CBP3, YAL035W, DRS1, NMD2, NOP5, RLF2*
	Protein of unknown function	*SCW11, YDL172C, YFR042W, YGR046W, YGR058W, YHR095W, YIL123C, YKL202W, YLR198C, YLR162W, YPL009C*
	Rescue, Defence, Apoptosis	*SIM1, SNF1*
	Transcription	*ASF1, CBF5, DRS1, EBP2, RLF2, SAS10, SIK1*
	Transport mechanism	*BFR2, SCT1*

Although an exact comparison of cross-hybridized filters to homologous hybridized filters was hindered as explained above, 38 *Z. rouxii* genes were monitored with a different trend in transcription behaviour upon salt stress compared to *S. cerevisiae*. *CLB5* did not significantly change upon NaCl-addition, whereas its expression was repressed upon NaCl-addition in *S. cerevisiae*. Table 2 shows a list of genes which behaviour differs upon salinity stress in the different yeast strains.

Table 2. List of *S. cerevisiae* genes, which have *Z. rouxii* homologous that show different up or down regulation following NaCl-stress. *Z. rouxii* gene expression ratios versus the ratios of *S. cerevisiae*. The gene expression was influenced by a salt shock of 1.4 M and 0.7 M, respectively. Expression ratios were scored as "down" or "up" when the genes showed a 3-fold in- or decrease in expression. The trend in ratio is given as *S. cerevisiae*/ *Z. rouxii*. Genes are ordered by their cellular function according to the MIPS Yeast Functional categories [22].

Ratio Trend *S. cerevisiae* vs *Z. rouxii*	Gene Function	Gene name
Down/ Unchanged	Biogenesis	*YEF3*
	Cell Growth, Cell Division and DNA synthesis	*CLB5, UTH1*
	Cellular Organisation	*DBP3, NPI46, PEP12, SNF1, SRP40, TFA1, TOP1, YEF3*
	Metabolism	*PDR16*
	Protein Synthesis	*FYV9, NPI46, PEP12, YEF3,*
	Protein of unknown function	*YGL164C, YHR131C, YOL054W, YOR309C*
	Rescue, Defence, Apoptosis	*UTH1, YRO2*
	Transcription	*DPB3, NOP58, PUF3, SRP40, TFA1, TOP1*
	Transport mechanism	*PEP12*
Unchanged/ Down	Protein of unknown function	*YDR474C*
Unchangedl/ Up	Cell Growth, Cell Division and DNA synthesis	*SIC1*
	Cellular Organisation	*BNI4, CTR1, HMO1, SEC7,SIC1, TFC5, RAD1*
	Ionic Homeostasis	*CTR1*
	Metabolism	*MET12*
	Protein synthesis	*BNI4*
	Protein of unknown function	*YPL216W*
	Rescue, Defence, Apoptosis	*RAD1*
	Transcription	*TFC5, YPR115W*
	Transport mechanism	*CTR1, SEC7*
Down/ Up	Cellular Organisation	*MDM12, SEN54*
	Metabolism	*THI6*
	Protein synthesis	*FPR4*
	Protein of unknown function	*YGL081W, YGR114C, YPL146C, YPL158C*
	Rescue, Defence, Apoptosis	*YKL088W*
	Transcription	*SEN54*

Convenience of cross-hybridization

Although much of the *Z. rouxii* genome has been sequenced [14], there are only 68 proteins elucidated and 44 partial and complete genes identified [19, 20]. None of these genes has a similarity greater than 90% to *S. cerevisiae* and none of them could be detected on the cross-hybridized filters. Concerning the absence of known *Z. rouxii* homologous genes, such as *ZrGPD1, ZrGPD2, ZrGCY1* and *ZrHOG1* on the filter, it is possible that they are not homologous enough or they exhibit an expression level below the detection limit. The first explanation is most likely in this case, since most of the known homologous genes missing on the filter are upregulated during NaCl- stress. Although identification of gene expression is a matter of both, homology to the spotted ORF and the abundance of its mRNA in the cell, the absence of *HOG1* on the GeneFilter® can be explained by the fact that the nucleotide sequences of *ZrHOG1* and *ZrHOG2* are 70 and 80 percent similar to those of *S. cerevisiae* [21]. This supports the idea that GeneFilters® are able to discriminate genes up to 83 percent homology [9]. For example, osmostress-sensitive genes of *S. cerevisiae*: *GPD1-GPD2* (75% identity), *GPP1-GPP2* (82%), and other isogenes up to 83% homology exhibited different spot intensities.

The filters showed identical spot values for isogenes like *ENO1-ENO2* (94%), *TDH1-TDH2* (90%), and others up to 100% identity, when hybridized with *S. cerevisiae* cDNA-probes. The value given for these genes is likely to be influenced by cross-hybridization of their isogenes. Therefore, GeneFilters® tend to cross-hybridize genes with a DNA sequence identity over 90%. This indicates that for *Z. rouxii* cross-hybridization, genes exhibiting over 90% homology to those of *S. cerevisiae* would encounter no difficulties in hybridization with *S. cerevisiae* ORFs.

In conclusion, GeneFilters® can hybridize *Z. rouxii* cDNA to the spotted *S. cerevisiae* ORFs when the genes are more than 83% homologous. The 155 monitored genes are therefore considered as true homologues of *Saccharomyces cerevisiae* genes. A blast search for these genes at the NCBI-databank revealed that until now, none of these genes have been identified in *Z. rouxii*.

Interestingly, among the novel *Z. rouxii* genes, 43 homologous to *S. cerevisiae* genes have been classified as hypothetical ORF or questionable ORF. It appears that they are highly conserved in these yeast species. Therefore, it is tempting to

speculate that they are highly conserved for an important, hitherto unknown, reason. It is worth pointing out that most of these unknown ORFs also displayed strong gene-activities.

The 86 genes involved in the responses to salt are associated with a variety of biological processes, catalogued according to the MIPS database [22]. Analysis of the data indicates that in many cases the response of *Z. rouxii* is different from that observed in *S. cerevisiae* cells. For example, several genes induced by salinity stress, play a role in cellular activity, such as protein synthesis, metabolism and cell growth. Induced cellular activity may suggest a tendency of this *Z. rouxii* strain towards halophility rather than osmotolerance. For instance, the response of gene *HMO1*, which is required for normal growth and for plasmid maintenance [23], is substantially upregulated after saline stress. This microarray data therefore, supports the indications of halophility in *Z. rouxii,* obtained by classical growth experiments. It has been reported that *Z. rouxii* grows better on media containing 5-23% NaCl than media containing no NaCl [24, 25]. By investigating the differences in gene expression and identifying additional genes influenced by NaCl it will be possible to further elucidate the salt tolerance by which *Z. rouxii* differs from *S. cerevisiae*.

Although the hybridization is dependent on DNA sequence-identity and results will be biased in favour of the most conserved genes, exposure of cDNA probes of *Z. rouxii* to *S. cerevisiae* GeneFilters® results in a transcriptional response. Obviously, genes that are unique within the salt tolerant yeast *Z. rouxii* will not be revealed by cross-hybridization. For example, random sequencing of the *Z. rouxii* genome revealed 203 potentially new genes with no known homology in *S. cerevisiae* [14, 15]. Nonetheless, GeneFilter® cross-hybridization has provided results that extend the knowledge of this yeast and its molecular behaviour under influence of NaCl. It allows us to generate hypotheses. In addition, groups of genes are elucidated in this organism, which would normally not have been selected for study. Cross-hybridization has provided us novel clues for genes present in *Z. rouxii* and demonstrates to be a rapid and easy detection method to gain an insight to genes and gene expression profiles in a yeast genus for which microarrays are as yet unavailable.

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CHAPTER THREE

Bat2p is essential in *Saccharomyces cerevisiae* for fusel alcohol production on the non-fermentable carbon source, ethanol

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In press

Abstract

Branched chain amino acids (BCAAs) are key substrates in the formation fusel alcohols, important flavour components in fermented foods. The first step in the catabolic BCAA degradation is a transaminase step, catalyzed by a branched chain amino acid transaminase (BCAAT). *Saccharomyces cerevisiae* possesses a mitochondrial and cytosolic BCAAT, Bat1p and Bat2p, respectively. In order to study the impact of the BCAATs on fusel alcohol production derived from the BCAA metabolism, *S. cerevisiae* BCAAT-deletion mutants were constructed. The BCAA L-leucine was exogenously supplied during cultivations with mutants of *S. cerevisiae*. *BAT1* deletion is not essential for fusel alcohol production under both, glucose- and ethanol growth conditions. The 3-methyl-1-butanol production rate of *bat1Δ*-cells on ethanol was decreased in comparison with wild-type cells, but the cells were still able to produce 3-methyl-1-butanol. However, drastic effects in fusel alcohol production were obtained in cells lacking *BAT2*. Although the constructed *bat2Δ*-single deletion strain and the *bat1Δbat2Δ*-double deletion strain were still able to produce 3-methyl-1-butanol, when grown on glucose, they were incapable to produce any 3-methyl-1-butanol, when ethanol was the sole carbon-source available. In the circumstances used, gene expression analysis revealed a strong upregulation of *BAT2* gene activity in the wild-type, when cells grew on ethanol as carbon-source. Apparently, the carbon-metabolism is able to influence the expression of BCAATs and interferes with the nitrogen metabolism. Furthermore, analysis of gene-expression profiles show that the expression of genes coding for other transaminases present in *S. cerevisiae* were influenced by the deletion of one or both BCAATs. Several transaminases were upregulated when a BCAAT was deleted. Strikingly, none of the known transaminases was significantly upregulated when *BAT2* was deleted. Therefore we conclude that the expression of *BAT2* is essential for 3-methyl-1-butanol formation on the non-fermentable carbon source, ethanol.

Introduction

Fusel alcohols are major organoleptic compounds in fermented foods, such as alcoholic beverages, soy sauce and bread. A metabolic pathway for the production of fusel alcohols has been proposed by Ehrlich [1]. The "Ehrlich pathway" involves three enzymatic activities (Fig. 1). Firstly, BCAAs are transaminated to the corresponding oxoacids by BCAATs. The ammonia binds to 2-oxoglutarate and the glutamate so formed can subsequently serve as a nitrogen donor for all other nitrogen containing compounds in the cell [4]. Secondly, branched chain oxoacids are converted to branched chain aldehydes via a decarboxylation reaction. Finally, the branched chain aldehyde is reduced to the corresponding fusel alcohol by an alcohol dehydrogenase [2, 7, 8].

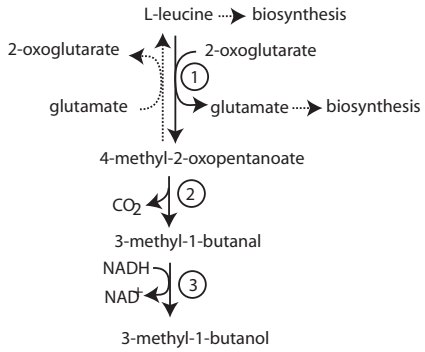

Figure 1. Schematic representation of the conversion of L-leucine, according to the Ehrlich pathway. 1) Branched chain amino acid transaminase 2) Branched chain oxoacid decarboxylase 3) Branched chain alcohol dehydrogenase.

Fusel alcohol formation in yeast also takes place when ammonia is used as a nitrogen source [8]. Carbon skeletons of fusel alcohols are then derived from the *de novo* synthesis of BCAAs through the isoleucine-valine-leucine (ILV) pathway and enter the Ehrlich pathway as branched chain oxoacids. The formation rate of fusel alcohols thus depends on the supply of BCAAs and on the supply of branched chain oxoacids.

In this study, we focus on the catabolic formation of fusel alcohols. Identification of two genes, *BAT1* and *BAT2* [9, 10], opened the way to more detailed studies of the first step of the Ehrlich pathway, the transaminase step. The deduced amino acid sequences of these genes show 77% identity to each other and between 30% to 50% to the homologous enzymes from plants (arabidopsis) and mammals [11]. Although these genes are responsible for the first step of the BCAA-degradation, it was found that deletion of a single *BAT* gene had no effect on fusel alcohol production [9,12]. Even the *bat1Δbat2Δ*-double deletion mutant was still able to produce some fusel alcohols on rich medium [13]. However, general consent exists that the two BCAATs genes, *BAT1* and *BAT2*, code for the most important mitochondrial and the cytosolic BCAAT, respectively. Our objective was to examine further the role of BCAATs in fusel alcohol production during food or beverage fermentation. In the first stage of food and beverage fermentation, *S. cerevisiae* quickly converts the available glucose to ethanol, which is subsequently utilized. Therefore, we studied the production of fusel alcohols in *S. cerevisiae* during growth on glucose and on ethanol. The effects of BCAAT-deletions were investigated on the fusel alcohol production level and on the gene-expression level by cDNA microarrays.

Materials and methods

Strains, media and genetic methods

The following isogenic *S. cerevisiae* strains were used in this study: VWk43 (CEN.PK113-7D) MAT **a**, as wild-type strain, *bat1Δ* single deletion strain (MAT **a** *ura3*-52 *bat1Δ*::*KanMX*), *bat2Δ* single deletion strain (MAT **a** *ura3*-52 *bat2Δ*::*URA3*) and *bat1Δbat2Δ* double deletion strain (MAT **a** *ura3*-52 *bat1Δ*::*KanMX bat2Δ*::*URA3*). For genetic procedures yeast cells were grown at 30°C, either on SD medium (2% glucose, 6.7 g 1(-1) Difco Yeast Nitrogen Base Without Amino Acids (YNB), or non-selectively in YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose). The *E. coli* strain used in this study was JM109 (*endA1, recA1, syrA96, thi, hsd*R*17, rk⁻, mk⁺ relA1 supE44*). Bacterial cells were grown in LB medium (1% yeast extract, 1.6% bacto tryptone, 0.5% NaCl); ampicillin (100 mg l(-1)) was added if necessary. Bacterial transformation and plasmid DNA isolation were performed according to procedures described previously [14].

Shake flask growth experiments were performed in minimal YNB medium (2% glucose and 1.67 g 1(-1) YNB w/o amino acids and ammonium sulphate) or YPD as described above. The media of uracil requiring strains were supplemented with 20 mg l(-1) uracil.

Disruption of BAT1 and BAT2

The *BAT1* gene disruption was performed by constructing a DNA cassette of the *KanMX* gene flanked by 40 bp sequences, identical to nucleotides -37 to +3 and +1180 to +1219 of *BAT1*. *KanMX* was cloned into pUC19 and the disruption cassette was generated by PCR, using the following primers for amplification: **5'l:** 5'-ACGCAAAATCAGCTAGAACCTTAGCTACTAAAAC-ATG-3' and **3'r:** 5'-TTTTTGGGGGGGGAGGGGATGTTTACCTTCATTA-TCATTA-3'. The 5' and 3' extremes from the *BAT2* gene were amplified by PCR using the following primers: **5'l:** 5'-GCCCAACATGCAACAAGAAC-3', **5'r:** 5'-**GGATCC**AACGGACATAGAAGAACGACAC-3' **3'l:** 5'CTTCTAT-GTCCGTT**GGATCC**TGTCGAAACCTGCATATC-3', and **3'r:** 5'-AGGTTG-AGATTGCCCTC TAA-3'. Primer 5'l has sequence identity with the *BAT2* gene from +4726 to +4745 and 5'r from +5193 to +5214. The bases pairs in bold correspond to an inserted *Bam*H I site. Primer 3'l was designed using the *BAT2* nucleotide sequence from 6712 to 6745. 3'r follows the sequence from 7136 to 7155. These two PCR products were ligated using the *Bam*H I site and cloned into the vector pUR2374 using *Hin*d III sites. The *URA 3* gene isolated from the vector YDp-U [15] was inserted between the PCR amplification products, using the *Bam*H I site. *bat1Δ* and *bat2Δ*-single- and *bat1Δbat2Δ*-double mutants were isolated and correct disruption of the *BAT1* and *BAT2* genes confirmed by PCR and sequence analysis.

L-Leucine bioconversion experiments

L-Leucine bioconversion experiments were performed using a Sixforce Multiple fermentor system (Infors, Bottmingen, Switzerland). Working volume of the bioreactors was 500 ml. Air was supplied at a flow rate of 20 l h(-1), while temperature was set at 30°C. The dissolved oxygen concentration was always above 25%. pH was maintained at 5.0 by automatic titration with 2 M NaOH, containing 1% (v/v) Struktol® as anti-foaming reagent. Stirrer speed was set at 500 rpm. Biomass was generated during controlled batch cultivation on glucose containing minimal medium (2% glucose, 6.7 g l(-1) YNB w/o amino acids) in

shake flasks. After the ethanol had been consumed, cells were washed with sterile deionised water and subsequently resuspended in minimal medium without a nitrogen source (2% glucose or 2% ethanol, 1.67 g l(-1) YNB w/o amino acids and ammonia). After equilibration for 10 min., L-leucine (37.5 ml of 26.6g l(-1)) was added to each 500 ml working volume, in order to obtain an initial L-leucine concentration of 15 mM. Samples were taken at appropriate time intervals and immediately filtered through a 0.22 μm filter. Off-gas analysis was performed using a PRIMA 600 gas analysis mass spectrometer (VG-gas, Winsford, Cheshire, UK).

Analytical procedures

Fermentation catabolites, ethanol and 3-methyl-1-butanol analysis were performed using high-pressure liquid chromatography (HPLC) analysis on an Aminex HPX-87H® column (300 x 7.8 mm, Biorad, Hercules, CA, USA) at 65°C. The HPLC column was eluted with 0.7 ml min(-1) 0.005 M H_2SO_4 and detection was performed with a Shimadzu RID-l0A refractive index detector and a Shimadzu SPD-M10A UV detector (195 - 340 nm, Shimadzu, Benelux Office s;Hertogenbosch, the Netherlands). Enzymatic determination of the initial and residual glucose concentration was performed using a Cobas Mira S (Roche Diagnostics Nederland B.V., Almere, the Netherlands) autoanalyser measuring $NADPH_2$ formation with an enzyme mix of hexokinase and glucose-6-phosphate dehydrogenase (Glucose HK 125, ABX diagnostics, Montpellier, France). Concentrations of L-leucine were determined using the AccQ tag ® (Waters, Milford Massachusetts, MA, USA) derivatisation method with a Waters HPLC system.

Microarray Analysis

mRNA was isolated from 10^6 cells, harvested from the Sixforce Multiple fermentor system (Infors, Bottmingen, Switzerland) after 240 minutes. Probe preparation, and hybridization to Yeast GeneFilters® (Invitrogen ResGen, Breda, The Netherlands) was performed as described previously [16]. All hybridizations and image analyses were performed in triplicate.

Data Acquisition and Analysis

Acquisition and quantification of array images and data filtering were performed using ImaGene® 4.2 software (BioDiscovery, Marina del Ray, CA, USA). The

background mean value was substracted from the signal mean. The resulting value was normalized against the total signal of the yeast gene set. The data was further analysed using Microsoft Excel 2000. For further statistical analyses to sift the variation in the triplicate measurements, Microsoft Excel Significance Analysis of Microarrays (SAM; v1.12) add-in was used [17]. The data representation used in Fig. 4 was generated using the Cluster and Treeview software [18].

Results

Fusel alcohol production during L-leucine bioconversion using glucose as carbon-source.

Two genes, *BAT1* and *BAT2* coding for BCAATs, are defined as key enzymes involved in the L-leucine degradation pathway. To investigate the impact of these genes on the fusel alcohol production, *S. cerevisiae* strains were constructed in which the genes coding for either the cytosolic (Bat2p), the mitochondrial (Bat1p) or both BCAATs were deleted. Correct disruption of the *BAT1* and *BAT2* genes was confirmed by PCR (data not shown). The conversion of L-leucine to the fusel alcohol, 3-methyl-1-butanol, was studied under fermentative (glucose as carbon source) and respiratory conditions (ethanol as carbon source).

All the strains grew respiro-fermentative on glucose. Strains VWk43, *bat1Δ* and *bat2Δ* displayed similar growth in minimal media on glucose and L-leucine. The double mutant *bat1Δbat2Δ* was hampered in its growth and displayed half the growth rate of the other strains. After 180 minutes no residual L-leucine was present in the medium of the VWk43, *bat1Δ* and *bat2Δ* -strains. After 240 minutes no residual L-leucine was present in the medium of the *bat1Δbat2Δ* mutant.

An initial 3-methyl-1-butanol concentration of 66 - 100 (3-methyl-1-butanol μmol (g DW)(-1)) was measured in the medium of all strains, after10 minutes equilibration on glucose. 240 minutes after L-leucine addition, the 3-methyl-1-butanol concentration was increased to 620 - 664 (3-methyl-1-butanol μmol (g DW)(-1)). At that time, the specific 3-methyl-1-butanol production rate of VWk43 was 157 (3-methyl-1-butanol μmol (g DW)(-1) h(-1)). The specific 3-methyl-1-butanol production rate of the *bat1Δ*-deletion strain was 143 (3-methyl-1-butanol μmol (g DW)(-1) h(-1)), and the *bat2Δ* and *bat1Δbat2Δ*-

deletion strains exhibited the same specific production rate of 138 (3-methyl-1-butanol μmol (g DW)(-1) h(-1)). Cells were able to compensate for the loss of one or both transaminases, Bat1p and Bat2p (Fig. 2A). Since 3-methyl-1-butanol was detected prior to L-leucine addition, we concluded that 3-methyl-1-butanol was also produced *de novo* via branched chain oxoacids. Deletion of *BAT1, BAT2* or both genes did not lead to a reduced fusel alcohol production.

Fusel alcohol production during L-leucine bioconversion using ethanol as carbon source

The cells showed a different behaviour in 3-methyl-1-butanol production when grown on ethanol (Fig. 2B). No initial 3-methyl-1-butanol was detected in the medium for all the 4 strains. After 240 minutes a residual L-leucine was measured in the medium 5-6 mM for VWk43 and the *bat1Δ* strain. A residual L-leucine concentration of 10 -11 mM was measured for the *bat2Δ* - and the *bat1Δ bat2Δ* strain.

3-Methyl-1-butanol was for the first time measured in the medium of VWk43 at t = 120 minutes. In the *bat1Δ*-strain, 3-methyl-1-butanol was detected at t = 150 minutes. After 150 minutes the VWk43 cells produced 3-methyl-1-butanol with a rate of 48 3-methyl-1-butanol μmol (g DW)(-1) h(-1). In the *bat1Δ*-strain a specific isoamyl production rate of 17 3-methyl-1-butanol μmol (g DW)(-1) h(-1) was achieved. Strikingly, no 3-methyl-1-butanol was produced by either the *bat2Δ*-strain or *bat1Δbat2Δ*-double deletion strain. This difference in behaviour between the *bat1Δ*-strain and the *bat2Δ*-strain or the *bat1Δbat2Δ*-double deletion strain, indicates that *BAT1* and *BAT2* have different characteristic properties in the cell. Obviously, the function of one BCAAT, Bat2p, cannot simply be taken over by the other BCAAT, Bat1p.

Analyses f expression of genes involved in the Ehrlich pathway

The role of BCAATs in the fusel alcohol production and the effect of the different carbon-sources in *S. cerevisiae* were further examined by genome-wide transcript expression analyses. We compared ethanol grown cells with glucose grown cells using Yeast GeneFilters®. The yeast strains were harvested 240 minutes after the L-leucine pulse, as described under materials and methods. Due to difference in carbon source, 1814 genes of the 6144 genes spotted on the filter were altered more than 2-fold in gene-expression in *S. cerevisiae* wild-type strain. The *bat1Δ*-strain showed altered expression for 1698 genes, when

comparing glucose grown cells to ethanol grown cells. And the *bat2Δ*- and *bat1Δbat2Δ*-strain showed 1740 and 1578 genes with an altered expression level, respectively.

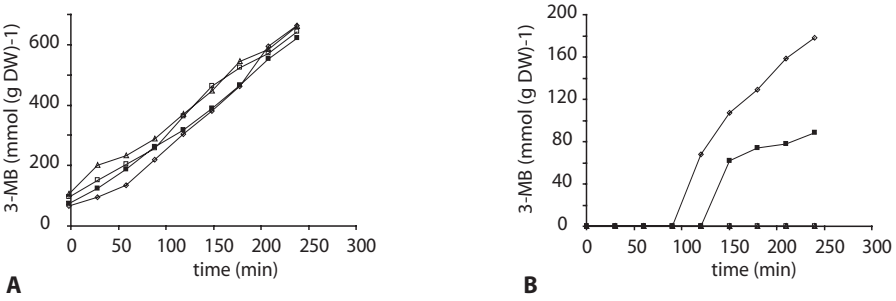

Figure 2A-B. Specific 3-methyl-1-butanol (3-MB) production in *S. cerevisiae* after a L-leucine pulse to the cells. VWk43 (◇), *bat1Δ*-deletion strain (■), *bat2Δ*- deletion strain (□), *bat1Δbat2Δ*-deletion strains (△). The concentration 3-methyl-1-butanol was measured each 30 minutes. A) Cells were grown on minimal medium with glucose as carbon-source and L-leucine as sole nitrogen-source. Experiments were performed in duplicate and differed less than 15%. B) Cells were grown on minimal medium with ethanol as carbon-source and L-leucine as sole nitrogen-source. Experiments were performed in duplicate and differed less than 15%.

Among the genes affected by the carbon-source were the genes of interest, *BAT1* and *BAT2*. Gene-expression of *BAT1* was observed in the VWk43 strain when cultivated on glucose. *BAT1* expression was also observed in the *bat2Δ* strain, during growth on glucose. In VWk43, the level of *BAT1* mRNA was diminished by a factor 5, by growth on ethanol when compared to glucose.

When the *bat2Δ* strain was cultivated on ethanol, *BAT1* expression was further reduced: its expression decreased by 13-fold (Fig. 3).

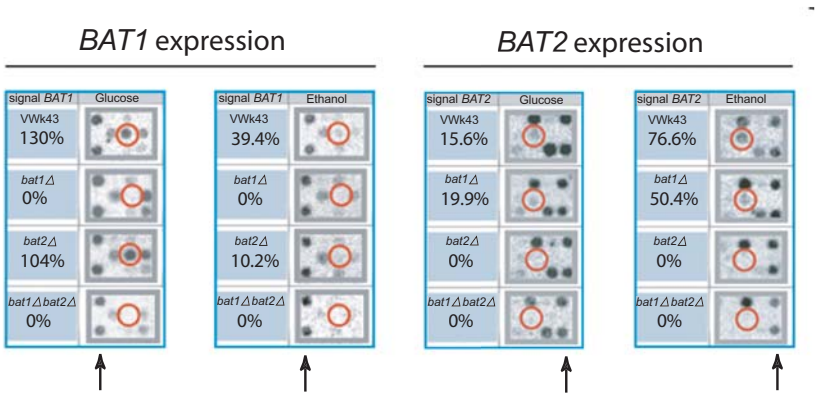

Figure 3. *BAT1* and *BAT2* expression on the Yeast GeneFilter® after 240 minutes of the L-leucine pulse. Arrows indicate the control spots, included as reference (100%) for the hybridization results. *BAT1* gene-expression was observed in glucose grown cells, except for the strains where *BAT1* was deleted. A low *BAT1* expression was detected when cells used ethanol as carbon-source. *BAT2* gene-expression was observed in ethanol grown cells, except for the strains where *BAT2* was deleted. Also, low expression of *BAT2* was observed in cells grown on glucose.

BAT2, encoding the cytosolic BCAAT, showed an enhanced gene-expression in VWk43 cells grown on ethanol. *BAT2* gene expression was 5-fold upregulated, compared to glucose grown cells. In the *bat1Δ*-strain, *BAT2* expression was also observed. In the *bat1Δ*-strain, *BAT2* expression was upregulated by growth on ethanol by a factor 2.5 (Fig. 3). These genes encoded enzymes responsible for the first step in the Ehrlich pathway. The strong expression of *BAT2* on ethanol is also confirmed by genome-wide gene expression studies of yeast cells in response to environmental changes [24]

The next step of the Ehrlich pathway involved the decarboxylation of 4-methyl-2-oxopentanoate, yielding 3-methyl-1-butanal. The group of genes coding for decarboxylases, which might be responsible for this step include *ARO10, LPD1, PDA1, PDB1, PDC1, PDC5, PDC6, PDX1* and *THI3*. The expression profile matrix for genes, which may be involved in the Ehrlich pathway, is shown in Fig. 4. *ARO10,* encoding for primary phenylpyruvate decarboxylase, minor 4-methyl-2-oxopentanoate carboxylase and putative indole-3-pyruvate decarboxylase [7, 19] was strongly expressed in all strains, cultivated on either glucose or ethanol. *LPD1* encoding dihydrolipoamide dehydrogenase, which is an component of pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex, branched chain 2-oxoacid dehydrogenase complex and glycine carboxylase complex [20, 21], was not significantly expressed in VWk43 cells during growth on L-leucine and glucose. The gene displayed a variable expression level, when grown on ethanol. However, expression of *LPD1* was unambiguously observed when *BAT2* was deleted (Fig. 4). *PDX1*, pyruvate dehydrogenase complex protein X, the binding protein for Lpd1p [22], exhibited the highest gene activity in the VWk43 cells during growth on glucose and in the *bat1Δbat2Δ*-double deletion mutant, during growth on ethanol. *PDA1,* coding for a subunit of a pyruvate dehydrogenase complex [20, 22] and the pyruvate decarboxylases *PDC1* and *PDC5* [23], transcription activities were not significantly influenced by deletion of a single BCAAT. The double deletion mutant of BCAAT on ethanol showed no *PDA1* expression. *PDC1* showed high expression levels in glucose grown cells and lower levels on ethanol (Fig. 4). The gene product of a pyruvate decarboxylase like enzyme, encoded by *THI3,* is proposed to play a specific role in the decarboxylation of branched chain oxoacids [6, 19]. Transcripts of this gene were detected under all conditions except in the *bat2Δ*- and *bat1Δbat2Δ*- strains, grown on ethanol (Fig. 4).

The last step of the Ehrlich pathway is the reduction step via an alcohol dehydrogenase. In this final step, 3-methyl-1-butanol is formed from 3-methyl-1-butanal [8]. Genes, coding for possible enzymes responsible for this reduction step are the *ADH* (alcohol dehydrogenase) genes and *ADH*-like genes. Looking at gene expression, no transcripts of the genes *ADH3, ADH6, ADH7* were observed in the VWk43 strain. *ADH6 (YMR 318c)* and *ADH7 (YCR105w)* were recently proposed to be involved in the synthesis of fusel alcohols [25]. *ADH1,* coding for a cytosolic isozyme, responsible for production of ethanol during fermentation, was clearly expressed in all strains, except for the *bat2Δ* strain grown on ethanol (Fig. 4).

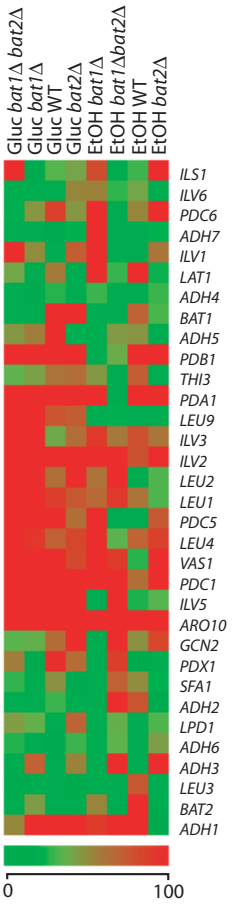

Figure 4. Expression matrix of genes, involved in the Ehrlich pathway. Strongly expressed genes are visualized in red, no detectable gene-expression is visualized in green. Other gene-expression values are visualized in colours between red and green. The expression matrix is produced by Cluster and Treeview [18] (see colour version at page 134).

Effects of carbon source and BAT1 and BAT2 deletion on the expression of genes involved in amino acid transamination.

The facts that *bat1Δ* and *bat2Δ,* and the *bat1Δ bat2Δ*- double deletion mutants behaved like VWk43 cells when grown on glucose, and that the *bat1Δ*-deletion strain was still able to produce fusel alcohols when grown on ethanol as carbon source, indicate that other enzymes are able to compensate for the loss of BCAATs under these conditions. *S. cerevisiae* posses several transaminases. According to Yeast Proteome Database [26], 23 identified transaminases or transaminase-like enzymes are present in the yeast cell. The expression patterns of several transaminase-encoding genes were influenced by the deletion of one or both BCAATs. A more than 2-fold change in expression level was used as threshold, comparing to VWk43. As described above, during fermentative growth on glucose, a clear *BAT1*-expression was observed in VWk43 and in the *bat2Δ*-deletion strain (Fig. 3). When *BAT1* was deleted in glucose fed cells, 4 of the 23 transaminases were significantly upreglated: *GFA1* (coding for a glutamine-fructose-6-phosphate transaminase), *SNO1* and *SNO3* (both coding for a putative pyridoxine (vitamin B6) biosynthetic enzyme with similarity to glutamine aminotransferases), and *YLR089c* (coding for an uncharacterized ORF, putatively involved in the biosynthesis and degradation of alanine by transferring the amino-group of glutamate to pyruvate, yielding 2-oxoglutarate and alanine) (Fig. 5A). When *BAT2* was deleted, upregulation of 5 genes encoding transaminases was observed, *GFA1, ARO9* (coding for an aromatic amino-acid transaminase), *BNA3* (coding for an enzyme which has similarity to human tyrosine transaminase), *SNO1* and *YLR089c* (Fig. 5A). When both *BAT1* and *BAT2* were deleted, *ARO9, BNA3, GFA1, SNO1* and *SNO3,* were significantly upregulated, compared to VWk43 (Fig. 5A). Together with the measured quantities of fusel alcohols under these condtions, the enhanced expression of the transaminases indicate strongly, that the deletion strains may use these enzymes to deaminate L-leucine during growth on glucose.

In ethanol-fed cells, *BAT1* deletion caused an upregulation in expression of the genes *HIS5* (coding for histinidol-phosphate amino transaminase)*, SER1* (coding for a phosphoserine transaminase, which catalyses the reaction 3-phosphohydroxy-pyruvate to 3-phosphoserine), and *SNO3* (Fig. 5B.) When *BAT2* was deleted the expression of the 23 genes coding for transaminases appeared to be unaffected. When both *BAT1* and *BAT2* were deleted, the gene coding for *AAT1* (a putative aspartate aminotransferase) showed a significant upregulation. (Fig. 5B).

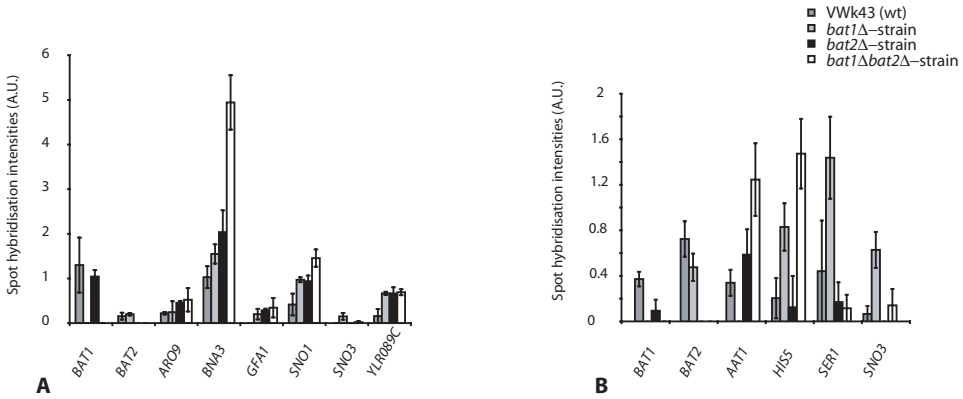

Figure 5A-B. Expression profile of transaminases, upregulated upon *BAT1, BAT2* or *BAT1* and *BAT2* deletion. A) Expression profile of upregulated transaminases in cells grown on glucose B) Expression profile of upregulated transaminases in cells grown on ethanol.

BAT1 and BAT2 gene clusters

Not only genes coding for transaminases, but many other genes were affected by the deletion of either *BAT1, BAT2* or both. However, a few genes displayed a similar transcription profile like the BCAATs. The average of three triplicate genome-wide transcripts for each condition was used to cluster. Hierarchical clustering revealed a similarity matrix consisting of rows (representing mean measurements for each gene) and columns (representing the different strains). The rows and columns were reordered according to the dendrograms produced by Treeview (Eisen, 1998). Treeview revealed a specific *BAT1* and a specific *BAT2* cluster (Fig. 6A, B).

The *BAT1* cluster consists of 24 genes (Fig. 6A). The 24 genes in the *BAT1* cluster displayed a low activity in the *bat1Δ* strain when grown on ethanol, and were highly expressed in *bat2Δ*-strain and VWk43 on glucose. Six of the 24 genes are coding for unknown proteins: *YPR050c, YGL182c, YDR179w-a, FYV12, YGR210c, YAR070c.* The other 18 genes belong to categories including metabolism, cell growth, cellular transport and energy.

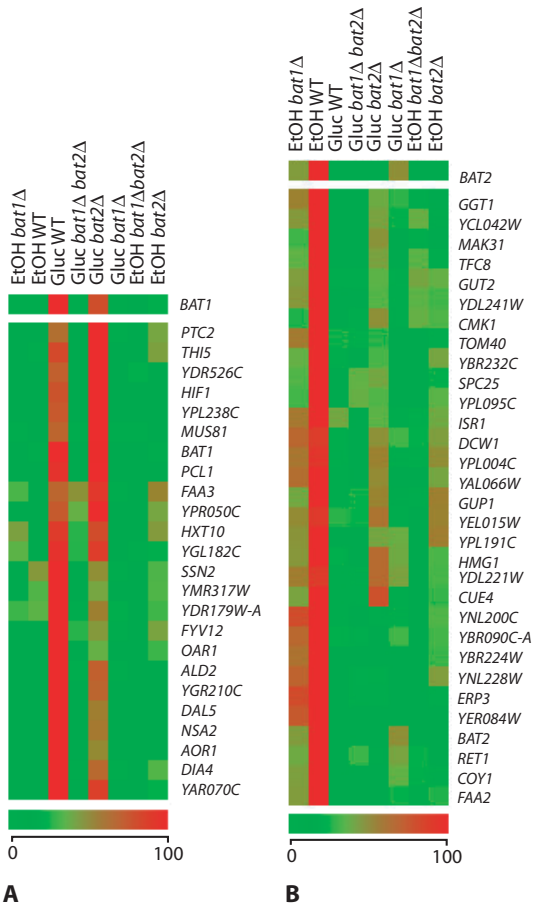

Figure 6A-B. Average-linkage clustering. A) *BAT1* similarity matrix. All 24 genes in the *BAT1* cluster are strongly down regulated in the *bat1Δ*-mutant by growth on ethanol, compared to VWk43 grown on glucose. B) *BAT2* similarity matrix. *BAT2* cluster consists of 31 genes. These genes in the *BAT2* cluster are strongly down regulated when *BAT2* was deleted and when grown on glucose in comparison to VWk43 on EtOH. Both clusters consist for 50% of genes coding for unclassified proteins. In contrast to the *BAT1* cluster, the *BAT2* cluster is dominated by genes, which possess STRE elements (see colour version at page 135).

The *BAT2* cluster consists of 31 genes (Fig. 6B). The activity of the genes in the *BAT2* cluster is low in the *bat2Δ* and the *bat1Δbat2Δ* strain when grown on glucose, and high in the ethanol grown VWk43. Remarkably, 21 of the 31 genes possessed one or more stress responsive elements (STREs) in their promoter region. The STRE, which has the consensus core sequence CCCCT, has been found to mediate stress-induced transcription [38]. In 12 genes, 1 STRE was found and the other 9 genes possessed at least 2 STREs. Among these genes are: *BAT2* (2 STREs in its promoter region (–1000, -1)), *GTT1,* glutathione transferase (3 STREs), *GUT2,* glycerol-3-phosphate dehydrogenase (1 STRE), CUE4, member of the CUE domain family, which bind ubiquitin-conjugating enzymes (6 STREs) and the gene *NHP6B,* coding for a protein with DNA-binding and DNA-bending activity, involved in transcriptional activation of a number of genes (4 STREs in its promoter region). Only 10 genes in the *BAT1* cluster had STREs in the promoter region. 6 genes possessed 1 STRE, and 4 genes possessed 2 STREs. *BAT1* itself has no STRE elements in the promoter region (-1000, -1).

It has been demonstrated previously that STREs mediate induction of transcription by ethanol stress [27]. This may explain the co-induction of the large number of genes, in their promotor possessing S*TRE* elements region, including *BAT2*.

Discussion

The events leading to the breakdown of L-leucine to fusel alcohols are not fully understood at present. The most well defined step in the L-leucine catabolic pathway is the first step, the transamination, catalyzed by BCAATs, encoded by *BAT1* or *BAT2* [9, 10, 12, 13]. Both isozymes are able to catalyze the transfer reaction of the nitrogen group from L-leucine, or if available isoleucine and valine, to 2-oxoglutarate, yielding a branched chain oxoacid and glutamate. BCAATs are not essential for fusel alcohol production by *S. cerevisiae* grown on glucose [13]. However, insufficient emphasis has been laid on variations in growth conditions, since most studies on Bat1p and Bat2p were focused on fermentative growth on glucose. Batch processes are mostly used in food and beverage fermentations. The environment for the micro-organisms in such processes is highly dynamic, and *S. cerevisiae* can switch from fermentative growth in an environment with excess of sugar, to respiratory growth when

sugar becomes limited [28]. Therefore, we studied the impact of BCAATs on fusel alcohol production on fermentative growth on glucose as well as on respiratory growth on ethanol.

Our results demonstrate that the highest fusel alcohol concentration is produced by *S. cerevisiae* during growth on glucose. Less of the L-leucine by-product, 3-methyl-1-butanol, is formed during growth on ethanol. An explanation for this observation could be that the cells apply different strategies on the different media. When growing on glucose, the strategy of yeast is to consume sugars in a rate as fast as possible. Due to the high glycolytic flux, there is a high turnover of the co-enzyme NAD^+ to NADH [37]. In order to maintain the internal NAD^+/NADH ratio, it is essential to reoxidise NADH back to NAD^+ as well. Therefore each putative electron acceptor will be used to regenerate NAD^+. As a result, various fermentative products, like ethanol are produced. The last step in the catalytic breakdown of L-leucine includes a reduction step. The remaining L-leucine carbon skeletons can function as an electron acceptor. The reduction of the aldehyde leads to the formation of the fusel alcohol, 3-methyl-1-butanol. However, when growing on ethanol, the yeast's strategy will be more focused on using reduction equivalents efficiently, since less NADH is available. Production of 3-methyl-1-butanol will mainly be driven by the necessity to use L-leucine for the nitrogen supply.

Furthermore, we have shown that Bat2p is essential for fusel alcohol production when cells are grown on ethanol. When *BAT2* was deleted, no 3-methyl-1-butanol was produced by the *bat2Δ* - and *bat1Δ bat2Δ*-deletion strains (Fig. 2B). Utilization of non-fermentable carbon-sources requires the participation of the glyoxylate cycle for the complete oxidation of ethanol to carbon dioxide and water [23]. Since there is less glucose converted to ethanol in the *batΔ*-deletion strains in comparison to the VWk43-strain, there is a possibility that these deletion strains also exhibit a slower metabolic activity when grown on ethanol. One of the consequences then would be a reduction in the production of 2-oxoglutarate, a metabolic intermediate from TCA cycle. This molecule is the acceptor of the ammonia group from the L-leucine molecule (Fig. 1). A strong reduction in the intracellular 2-oxoglutarate concentration may lead to a shortage of nitrogen-acceptors, since L-leucine is the only nitrogen source available. This could be a plausible explanation for the reduced 3-methyl-1-butanol production on ethanol, but it does not fully explain the incapability for the *bat2Δ*-strains to produce any fusel alcohols.

cDNA microarrays displayed a strong *BAT2* gene expression upon exposure to ethanol. Low gene-activity for *BAT2* was observed in cells cultivated on glucose. This is in agreement with the unaffected fusel alcohol production in cells grown on glucose, which lack the *BAT2* gene. On ethanol however, deletion of *BAT2* caused a severe effect on the fusel alcohol production. In the *bat2Δ*-deletion strain cultivated on ethanol, no gene-activity for *THI3,* which is likely to be involved in the decarboxylation of 4-methyl-2-oxopentanoate, was detected [6]. This suggests that when the first step of the Ehrlich pathway is blocked due to *BAT2*-deletion, the second step may be blocked by the termination of expression of the gene *THI3*. For the final step in fusel alcohol synthesis, a novel NADP-dependent branched chain ADH (bcADH) has been detected, which is able to reduce 3-methyl-1-butanal to 3-methyl-1-butanol under anaerobic growth conditions [29]. Two genes, *YMR318c* (*ADH6*) and *YCR105w* (*ADH7*), have been identified to encode for NADP(H)-dependent alcohol dehydrogenases [25, 30]. Although, no expression of these genes was detected in wild-type cells grown on glucose or ethanol. We only found a correlation for *ADH1*. *ADH1* was clearly expressed in wild-type cells and showed a strong down-regulation in the *BAT2*-deletion strain, grown on ethanol.

Although the yeast possesses two highly homologues BCAAT isozymes Bat1p and Bat2p, *BAT1* and *BAT2* have different expression patterns in *S. cerevisiae* during fusel alcohol production. Also the *bat1Δ* and *bat2Δ*-deletion strains behaved differently compared to each other. In wild-type cells, *BAT1* was strongly expressed in glucose grown cells, while a strong *BAT2* expression was found in ethanol grown cells. When one of both of the BCAATs was deleted, cells were still able to produce fusel alcohols during growth on glucose. On ethanol, on the other hand, when *BAT2* was deleted fusel alcohol production was stopped. These observations suggest that other enzymes are able to take control over the transaminase step during growth on glucose, whereas on ethanol Bat2p appears to be essential for the forming of branched chain alcohols. When *BAT1* was deleted in cells grown on glucose, 4 genes coding for transaminases, including *GFA1, SNO1, SNO3* and *YLR089c* showed enhanced gene-expression in comparison to VWk43 cells (Fig. 5A). It is worth noting that deletion of *BAT1,* which encodes for a mitochondrial transferase [9], causes an upregulation of a transferase, *GFA1,* which is located in the cytoplasm [32]. The localization of the other three transferases is not elucidated, yet. In this case, a cytosolic transaminase would then be backup for a mitochondrial transferase. This is a possibility, since L-leucine can be directed from the mitochondria to the cytosol. Deletion of *BAT2* caused, in glucose grown cells, an upregulation in

gene-expression in the following 5 genes coding for transaminases: *ARO9, BNA3, GFA1, SNO1,* and *SNO3* (Fig. 5A). Of these 5 genes, the gene-products of *ARO9,* and *GFA1* [32, 33] are localized in the cytosol. Two genes coding for cytosolic transferases *HIS5* and *SER1* [33] and one gene coding for a transerase *SNO3* [34], which cellular localization is unknown, showed an enhanced expression in *bat1Δ* cells, cultivated on ethanol. When *BAT2* was deleted in ethanol grown cells, no significant enhanced gene expression of other transaminases was observed.

The L-leucine bioconversion experiments, combined with the gene-expression data, indicate that upon deletion of one or two of the BCAATs, L-leucine transamination predominantly takes place in the cytoplasm. Previous experiments indicated that catabolic L-leucine transamination is likely cytoplasmic [35, 36]. Data available on the web (http://www-genome.stanford. edu/yeast_stress) indicate that *BAT2* was upregulated during various stress conditions [24]. The cluster derived from gene expression profiling revealed that *BAT2* was co-expressed with several genes containing one of more *STRE*-elements (Fig 6B). *BAT2* itself possesses 2 *STRE*-regulatory elements. One could speculate that these *STRE*-regulatory elements may therefore be involved in the regulation of *BAT2*. Further experiments on those elements would be necessary to elucidate this hypothesis. It could be interesting to delete one or both *STREs* in the *BAT2* promoter region, to test the influence of these regulatory elements.

If an alternative mechanism for fusel alcohol production exists in yeast, as suggested previously [2, 5] this mechanism is apparently not capable of rescuing cells from the *BAT2* deletion, during growth on ethanol. However, our data do not exclude involvement of such an alternative route, in addition to the Ehrlich pathway (including pyruvate decarboxylase), in fusel alcohol production by wild-type *S. cerevisiae*. Especially as the double mutant strain *bat1Δbat2Δ* is able to produce fusel alcohols in a glucose-rich medium with L-leucine as the sole nitrogen source. We found also indications that another enzymes could transfer the amino group from L-leucine to an acceptor. Nevertheless, gene expression profiles also showed that upon *BAT2*-deletion during growth on ethanol there is no convenient backup gene for *BAT2*. Additionally, no 3-methyl-1-butanol was produced by the *bat2Δ*- and *bat1Δbat2Δ*- deletion strains. Therefore, we conclude that *BAT2* is indispensable for the production of fusel alcohols by growth on ethanol.

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CHAPTER FOUR

Genome-wide transcription survey on fermentative production of flavours in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae is widely used as aroma producer in the manufacture of fermented foods and beverages. Catabolism of branched chain amino acids (BCAAs) by this yeast contributes to aroma formation by the production of methyl-branched aldehydes and carboxy acids. During growth on L-leucine, 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoate and 3-methylbutyrate accumulate outside the cell. To study the underlying molecular mechanism of the formation of these compounds, we performed genome-wide transcription analyses on GeneFilter® arrays. The expression profile of yeast during flavour formation, when cultivated on L-leucine, was compared to the expression profile of cells cultivated on ammonia. In addition, the expression profiles of cells cultivated in batch cultures were compared to cells cultivated under continuous growth conditions. Under both conditions, L-leucine or ammonium sulphate was used as nitrogen source. Analysis of the transcriptome in these samples yielded a group of 117 genes, altered in gene expression under both cultivation conditions. Comparing genes from this group with genes altered in expression levels for batch or continuous culture (CSTR) cells, we found that genes encoding enzymes of different amino acid metabolism pathways were present in the group of 117 genes. Strikingly, the group of the BCAA metabolism was not significantly present as altered gene expression group. Genes, identified with altered expression levels in only batch or continuous cultures represented functional groups concerning energy, protein fate, cell cycle and DNA processing. Clustering of genome-wide data revealed that the type of cultivation overruled the differences in N-source in genome-wide gene expression profiles. This clearly emphasized the important role of the sample history in gene expression analysis. Furthermore, cDNA microarray data also showed up regulation of the genes *YGL059w* (branched chain 2-oxoacid dehydrogenase kinase) and *ACH1* when flavour compounds were formed. A functional branched chain 2-oxoacid dehydrogenase may explain the presence of the metabolite 3-methylbutyrate.

Introduction

During food fermentation processes, fungi are at the core of flavour formation. For example in production of bread and alcoholic beverages the yeast *Saccharomyces cerevisiae* is responsible for the characteristic aroma profile. The responsible products for this, like in all foods, consist of a complex mixture of flavour compounds. However, the characteristic impact of these fermented food flavours is determined by a prominent group, consisting of fusel alcohols and their derivatives. Commonly known fusel alcohols are the substances: 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol [1, 2].

To predict, control and enhance the production of these 'character-impact' compounds, profiling of yeast metabolic routes is of particular importance. When ammonia is used as nitrogen source, fusel alcohols are synthesized via the isoleucine-valine-leucine (ILV) pathway [30]. However, their concentration drastically increases when branched chain amino acids are present in the media. The dogma of fusel alcohol formation in yeast, the so-called "Ehrlich pathway", was founded a century ago [3]. The Ehrlich pathway assumes the conversion of the branched chain amino acids to fusel alcohols by three enzymatic steps. The first step is a transaminase step, from where the amino group of branched chain amino acids is transferred to 2-oxoglutarate, resulting in branched chain oxoacids and glutamate. The branched chain oxoacids are subsequently converted to branched chain aldehydes via a decarboxylation reaction. Finally, the branched chain aldehydes are reduced to fusel alcohols by an alcohol dehydrogenase.

There are several questions concerning the Ehrlich pathway and fusel alcohol production. Firstly, the molecular mechanisms behind the production of flavour compounds remain unclear. Enzymes, involved in the synthesis of these flavours have been identified biochemically, but the identity and genes encoding enzymes involved in this pathway, are not fully elucidated. The only well-defined genes in the Ehrlich pathway are *BAT1* and *BAT2,* encoding branched chain amino acids transaminases, responsible for the first step [4, 5, 6]. Both isozymes are able to catalyze the transfer reaction of the nitrogen group from leucine, or if available isoleucine and valine to 2-oxoglutarate. Although these genes were identified, they are not essential for fusel alcohol production by *S. cerevisiae* grown on glucose [7]. Recent microarray data indicate that the function of Bat1p and Bat2p differ, dependently on available carbon sources [8]. Concerning the

other Ehrlich pathway steps, it has been proposed that in particular PDC-like enzymes in *S. cerevisiae*, coded by *YDL080c* and *ARO10,* are involved by the decarboxylation of 2-oxopentanoate [9, 10]. A NADP-dependent branched chain Adh (bcADH) might be responsible for the reduction of the branched chain aldehyde to the fusel alcohol [11]. Two genes have been identified, *YMR318c* (*ADH6*) and *YCR105w* (*ADH7*), encoding NADP(H)-dependent alcohol dehydrogenases [12, 13]. However, clear evidence about the identity of the responsible *ADH* is not available.

The second inadequacy of the Ehrlich pathway is the generation of different oxo-acids and fusel alcohols from branched chain amino acids, which cannot be explained by a linear pathway. Prior to this research, no genes were identified to increase fusel alcohol production [14, 15]. With the development of cDNA microarrays, a tool has become available to identify or better understand the expression of genes that function in metabolic pathways or as regulatory genes for that pathway [16, 17].

This study was performed to generate tools for the identification of genes involved in fusel alcohol production, intended to elucidate fusel alcohol formation. Four flavour substances 3-methyl-1-butanol, 4-methyl-2-oxopenta noate, 3-methyl-2-oxobutanoate and 3-methylbutyrate were observed when yeast was cultivated in the presence of L-leucine. cDNA microarray data showed active genes of various pathways during 3-methyl-1-butanol formation. Genes involved in amino acid, amine, caboxylic acid - and organic acid metabolism were affected under circumstances in which flavour substances were produced. Gene targets of the transcriptional activator *GCN4* and *GLN3* were over represented in the cluster of genes and altered in response to flavour formation. In addition, microarray data revealed a significant upregulation of *YGL059w,* a ORF which has sequence similarity to human branched chain oxoacid dehydrogenase kinase. The BC oxoacid dehydrogenase complex could be responsible for the formation of flavour acids such as 3-methylbutyrate.

Materials and Methods

Yeast strains, maintenance and shake-flask cultivations

The isogenic wild-type *S. cerevisiae* strain CEN-PK 113-7D was used. Frozen stock cultures containing 20% w/v glycerol were stored at -80°C. Working stocks were maintained on YP- agar slants containing 20 g/l glucose. Shake-

flask cultivation was performed in 250 ml Erlenmeyer flasks containing 100 ml medium. Cultures were incubated at 30°C in a rotary shaker (180 rpm). Cells were pregrown overnight on non-selectivity YPD medium, containing 2% glucose. Subsequently, 1 ml of the preculture was inoculated in mineral medium consisting of 1.7 mg/ml yeast nitrogen base without amino acids and without ammonium sulphate (YNB; DIFCO Laboratories, Detroit, USA), supplemented with 100 mM glucose 15mM L-leucine to an OD_{600} of 1.0 (± 0.2).

At this point cells were collected for determination of expression profiles. Samples were immediately snap frozen in liquid nitrogen after collection and stored at –80 °C.

Chemostat cultivations

Inoculum cultures were grown for approximately 48 hours in Erlenmeyer flasks at 30°C on YNB medium. The cell suspension (100 ml) was transferred to a 2 L BiofloIII fermentor (New Brunswick Scientific, Nijmegen, the Netherlands), connected to a computer controller unit running with Advanced Fermentation Software (New Brunswick Scientific, Nijmegen, the Netherlands). After overnight growth carbon-limited chemostat cultivation was performed at a temperature 30°C and a stirrer speed of 800 rpm. The culture pH was maintained at 5.0 by automatically controlled addition of 1 M KOH. The yeast was grown at a dilution rate of 0.10 per hour with a feed containing 15 mM L-leucine and 20 g glucose per liter on EGLI culture medium [18]. Continuous feed was connected after overnight batch growth. The working volume was kept at 2 L by continuous removal of effluent and the airflow was also kept constant. Carbon dioxide production and oxygen consumption were monitored online by a Servomex 1440 Gas Analyser (Servomex B.V., Zoetermeer, the Netherlands). The ethanol production was monitored by a Servomex gasanalyser 2500 Single Beam Infrared. During cultivation samples for off-line analyses were taken from the bioreactor: 10 ml of a fresh sample was taken and centrifuged for 5 minutes at 4000 rpm, 5°C. The supernatant was used for analysis of extracellular substrates and metabolites. The remaining pellet was used for determining the biomass dry weight. Fresh sample was also used for determination of the optical density and preparing samples for fusel alcohol measurements. For the latter 100 µl sample was taken and 300 µl methyl formate together with a few grains of sodium chloride were added. Every time a sample was taken, the dilution rate, the pH and the purity of the culture were kept constant (Table 1).

Table 1. CSTR data of cultures grown on ammonium sulphate and cultures grown on L-leucine. Samples were taken in duplicate, after the bioreactor was refreshed at least two times its own volume.

V (l)	D(1/h)	Glucose (g/l)	NH_4 mM	NH_4 res mM	Leu mM	Leu res mM	Dwt (g/l)
1.92	0.21	7.13	7.25	4.59	0	0	1.50
1.98	0.18	6.85	0	0	16.02	12.08	1.42

Determination of culture dry weight and cell amounts

For the biomass dry weight determination the pellet, obtained as described above was washed with demineralized water and centrifuged again. Next, the pellet was dried overnight at 80°C. The optical density (OD_{600}) of the medium was determined on a spectrophotometer (Ultrospec 3000, Amersham Biosciences, Roosendaal, the Netherlands).

Substrate and metabolite analysis

The amount of glucose present in the reservoir media and supernatant was enzymatically determined a Cobas Mira S (Roche Diagnostics Nederland B.V., Almere, the Netherlands) autoanalyser measuring $NADPH_2$ formation with an enzyme mix of hexokinase and glucose-6-phosphate dehydrogenase (Glucose HK 125, ABX diagnostics, Montpellier, France). 3-methyl-1-butanol concentrations were determined by gas chromatography with flame ionization detector (GC 8000 Top equipped with two autosamplers in order to double the number of samples that can be handled at the same time; CE Instruments, Thermoquest, Milan, Italy), using on-column injection and He as carrier gas. Columns used were Zebron ZB-1 (30 m, ID 0.32 mm, 0.50 μm film, Phenomenex, Torrance, CA, USA, and DB-WAX (30 m, ID 0.53 mm, 1.00 μm film, J and W Scientific, Folsom, CA, USA). Samples were extracted with methyl formate (1:3), containing 1-hexanol or 1-pentanol as internal standards. Methods were optimized for fast separation by using short temperature gradients to increase the throughput of samples (retention times of 5 min or less).

Concentrations of leucine were determined using the AccQ tag® (Waters, Milford Massachusetts, USA) derivatisation method with a Waters HPLC system.

Cell Extract Preparation and Synthesis of cDNA

mRNA, from 10^6 cells, harvested from batch cultures at t=24 h or from continuous culture steady state samples and was isolated as previously described [19]. The mRNA quality was determined using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) according to the manufacturers protocol. The small amount of mRNA from each sample was loaded on a microgel, electrophoresed, scanned and analyzed for the quantity and integrity of the 18s and 28s ribosomal RNA bands, to ensure that the same amount of mRNA was examined for all samples. The next steps, the synthesis of [^{33}P]CTP-labeled cDNA and GeneFilter® (Invitrogen ResGen, Breda, The Netherlands) hybridization was performed as previously described [19].

Data analysis and spot validation

Images were scanned at 50 micron resolution in ImageQuant and were imported into the ImaGene® microarray analysis software (BioDiscovery Inc.,El Segundo, CA, USA). ImaGene® was used for a preliminary quantification of spot intensities to compare gene filter images pair wise. In this way the genes that were differentially expressed were identified. To determine the extent of induction or repression of gene expression, all spot intensities were normalized against the total spot. The relative mRNA levels calculated in this way indicate whether a gene is strongly or poorly expressed.

Each appearing genespot was averaged and validated. The web-accessible gene expression analysis tool SNOMAD (Standardization and NOrmalization of MicroArray Data) was used to correct for bias and variance which are non-uniformly distributed across the range of microarray element signal intensities. Two non-lineair transformation were performed for the local mean normalization and for local variance correction (Z-score generation using a locally calculated standard deviation), in order to correct hybridization artifacts resulting from the radioactive hybridization + washing process (manifested as non-uniform background intensities) [20]. Microarrays were performed in duplicate. To identify significantly altered gene expression and reduce the presence of false positives the program Significant Analysis of Miroarrays was used [21]. This program compared the difference in gene expression within the duplicates to that of the other samples. Ammonium batch cultured cells were compared with L-leucine batch cultured cells and the ammonium continuous cultured cells were compared with L-leucine continuous cultured cells.

Results

Growth and flavour formation in batch and continuous cultures

S. cerevisiae was grown in 0.5 L fermentors on mineral medium and 100 mM glucose. Batch growth was followed during 7 days. The branched chain amino acid, L-leucine, was used as nitrogen source. Control cells were cultivated, using ammonium sulphate as nitrogen (N)-source. Cells started to grow exponentially after 24 h. After 92 h the cells grown on ammonium sulphate reached an OD_{600} of 7.8. Yeast cultivated on L-leucine as N-source reached an OD_{600} of 6.9. There was no significant difference in lag-phase between the cultures. L-Leucine was totally consumed at t=92 h. After 92 h, a residual glucose concentration was measured for the L-leucine- or ammonium sulphate cultivated cells of 2.9 or 3.6 mM, respectively (Fig. 1).

Four volatile flavour substances; 3-methyl-1-butanol (isoamyl alcohol), 4-methyl-2-oxopentanoate (α-keto isocaproate), 3-methyl-2-oxobutanoate (α-keto isovalerate) and 3-methylbutyrate (isovaleric acid), were detected in the supernatant of L-leucine-cultivated cells (Fig. 2). Cells started to produce 3-methyl-1-butanol and 4-methyl-2-oxopentanoate immediately after inoculation. After 24 h the metabolite 3-methylbutyric acid was detected in the medium. After 48 h the highest 3-methyl-1-butanol concentration of 4.54 mM was measured. After 192 h the concentration had declined to 3.59 mM, most probably because of evaporation. The other metabolites, important for flavour determination due to their penetrating aroma, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid, and 3-methylbutyric acid reached concentrations of 1.05 mM, 0.14 mM and 1.38 mM, respectively. None of these products were detected in the supernatant of ammonium sulphate cultivated cells.

4-Methyl-2-oxopentanoate is regarded as the first product in the L-leucine degradation pathway, also referred in yeast as the 'Ehrlich pathway'. The detected 3-methyl-1-butanol is known as the final product of the L-leucine degradation pathway. The other detected products, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid, are not known to be involved in this pathway.

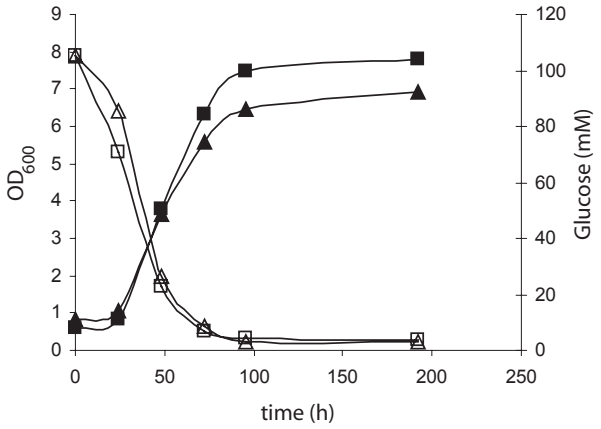

Figure 1. Growth curve and glucose consumption of *S. cerevisiae* cells, batch-cultivated in 0.5 L on minimal medium with 7g/l glucose. OD_{600} of cells cultivated on ammonium sulphate (■),OD_{600} of cells cultivated on L-leucine (▲), Glucose concentration of cells cultivated on ammonium sulphate (□), Glucose concentration of cells cultivated on L-leucine (△).

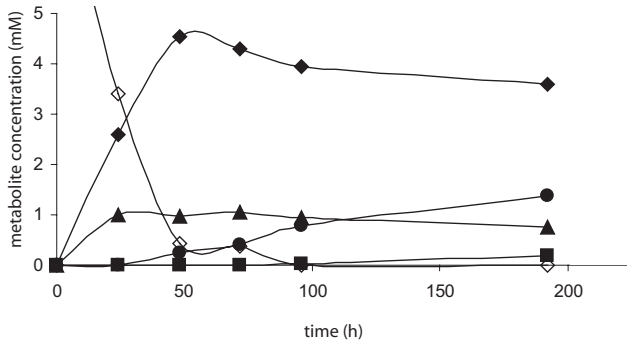

Figure 2. Production of volatile metabolites and L-leucine consumption of *S. cerevisiae* cells, batch-cultivated in 0.5 L on minimal medium with 7g/l glucose and 15 mM L-leucine. 3-Methyl-1-butanol (◆), L-leucine (◇), 4-methyl-2-oxopentanoate (▲), 3-methyl-2-oxobutanoate (■) and 3-methylbutyrate (●).

As in batch cultured cells, in CSTR-cultured cells, the same flavour substances, 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid emerged. A concentration was measured in the medium of 0.4 mM 3-methyl-1-butanol, 0.25 mM 4-methyl-2-oxopentanoate, 0.1 mM 3-methyl-2-oxobutanoic acid and 0.025 mM 3-methylbutyric acid (Fig. 3).

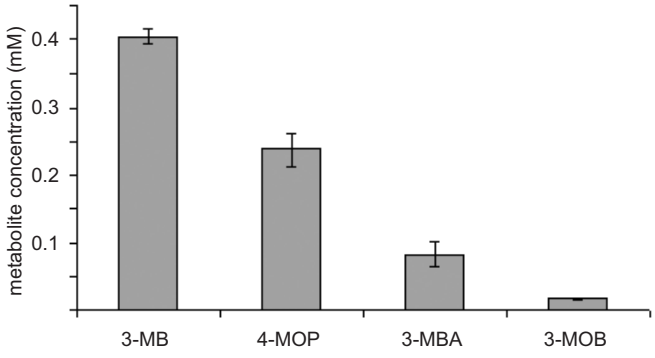

Figure 3 Steady state concentrations of metabolite measured in the medium of chemostat cultures with a working volume of 2 L and a dilution rate of 0.2 h^{-1}. Cells were cultivated on 15 mM L-leucine and 7 g/l glucose. 3-MB = 3-methyl-1-butanol; 4-MOP = 4-methyl-2-oxopentanoate, 3-MBA = 3-methylbutyric acid, 3-MOB = 3-methyl-2-oxobutanoic acid.

Also under these conditions no flavour substances were detected in the medium with ammonium sulphate as N-source. Cells were harvested for cDNA microarray analysis in the late exponential phase during batch growth, and in the continuous culture grown cells, after the bioreactor reached a steady state and was refreshed at least two times its own volume. The procedures were repeated to obtain independent duplicates. For each sample independent mRNA isolations were carried out and ^{33}P-labeled cDNA was synthesized. Labeled cDNA was hybridized with GeneFilters® containing 6144 yeast ORFs.

Genome-wide survey on gene expression of L-leucine vs Ammonium grown cells

Raw datasets were normalized by SNOMAD [20] for background correction and correction of error prone filter hybridization or spot imaging. After normalization, low expression values were removed and ^{2}log transformations were applied.

^{2}Log ratios of gene expression levels were analysed with the SAM-test [21] to identify genes significantly altered in gene expression ($p<0,01$ and $-1>{}^{2}\log(\text{ratio})>1$). In batch cultured cells 930 genes were identified with a significantly altered gene expression when cultivated on L-leucine, compared to that of cells cultivated on ammonia. In the other experiment, cells cultivated in a CSTR under controlled pH, aeration and dilution rate, 752 genes showed significantly altered gene expression. Of the 930 genes, 813 genes showed altered expression specifically in the batch-cultured cells and 117 genes showed similar altered expression in batch as well as in continuous cultured cells. Concerning the CSTR cells, 635 genes of the 752 genes showed altered expression, which were not found in the gene group of batch-cultured cells (Fig. 4).

The three gene groups, 635 genes of the continuous culture, 117 genes of both cultures and 813 genes of the batch culture were further categorized, according to the MIPS database [22]. These analyses revealed that most genes with affected expression levels have functions in cell cycle and DNA processing, energy, metabolism, protein synthesis or fate, rescue and defense, transcription, transport or are classified as proteins with unknown biological functions (Table 2).

Figure 4. Venn diagram of the influence of cultivation methods. 635 genes showed an altered expression pattern exclusively in CSTR-cultivated. 813 genes showed an altered expression pattern exclusively in batch cultivated cells. 117 genes showed affected expressions under both conditions (L-leucine versus cells cultivated on ammonia) .

Table 2. Gene groups with common biological functions according to MIPS database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html) among the transcripts that were altered in expression, comparing L-leucine grown cells with ammonia grown cells.

Function	Batch	CSTR	Overlapping
Cell Cycle and DNA Processing	73 (9%)	35 (6%)	2 (2%)
Energy	54 (7%)	48 (8%)	15 (13%)
Metabolism	137 (16%)	120 (18%)	28 (23%)
Miscellaneous	37 (5%)	77 (12%)	3 (3%)
Protein Synthesis or Fate	183 (22%)	81 (13%)	15 (13%)
Rescue and Defence	38 (5%)	43 (7%)	11 (9%)
Transcription	110 (13%)	49 (8%)	10 (9%)
Transport	79 (10%)	77 (12%)	9 (8%)
Unclassified Proteins	102 (13%)	105 (16%)	24 (23%)
Total	**813 (100%)**	**635 (100%)**	**117 (100%)**

Miscellaneous: cellular communication, signal transduction, regulation of interaction with cellular environment, cell fate or viral and plasmid proteins.

Further partitioning of genes associated with metabolism revealed that the majority of the overlapping metabolism genes, 82%, were specifically involved in amino acid metabolism.

To establish the significance of the presence of identified gene groups, SGD GO TermFinder was used [20]. 69 Gene ontology (GO) groups were identified with GO TermFinder as significantly represented function groups within the group of 117 genes ($p<0.01$). These functional groups included genes involved in the amino acid biosynthesis, amine biosynthesis, caboxylic acid - and organic acid metabolism (*ARG1, BAT1, ACH1, GCN4, LYS1, CIT1, CIT2, DLD3, ARO3, GCV1, HOM3, ARG3, ARO4, SER33, HOM2, PDA1, ARO8, LYS21, ARO2, GLN1, ARG4* and *TRP4*). Separately mentioned amino acid metabolism groups were the group of aromatic amino acids (*ARO3, ARO4, ARO8, ARO2* and *TRP4*), serine, homoserine and threonine (*GCV1, HOM3, SER33* and *HOM2*), arginine and glutamine (*ARG1, GLN3, GDH2, ARG3, GLN1* and *ARG4*).

The similar altered gene expression profiles in yeast under the two different growth conditions may point to similar functions under batch and continuous growth. Under both conditions, 4 volatile flavour substances were produced, correlating the appearance of he aroma compounds with the altered gene expression. As major affected gene expression groups consisted of genes encoding proteins involved in amino acid metabolism, not specifically for L-leucine, within the identified genes, this indicates that amino acid metabolism pathways, other than the BCAA pathways, play significant roles in the formation of volatile flavours, independently of growth rate or other variation in cellular environment.

Identification of Regulatory Elements

Genes with similar expression patterns often have common regulatory elements in their promoter region. These elements are bound by similar transcription factors. Concerning N-metabolism, several upstream activation sequence (UAS) elements have been discovered. UAS_{NTR} for example, consists of two separate dodecanucleotides sites with the sequence GAT(T/A)A at their core [23] and genes containing this element can be induced or repressed via this element. To detect over-represented elements in the promoter regions of the 117 selected genes, the program Regulatory Sequence Analysis Tools (RSAT) was used.

RSAT provided a series of modular computer programs specifically designed for the detection of regulatory signals in non-coding sequences [24]. Two regulatory elements were significantly present within the group of 117 genes; the *GCN4* element (GAGTCA; $p=3.3^{-5}$) and the *GLN3* element (GAT(T/A)A; $p=2.5^{-5}$). The transcription factor *GCN4* was descibed as regulator in the general response to amino acid starvation [25]. *GCN4* was upregulated by growth on L-leucine compared to ammonia and showed a more than 4-fold upregulation in the batch and continuous culture. *GLN3,* encodes a GATA-type zinc finger transcription factor for positive N- regulation [26]. The *GLN3* gene is required for the activation of transcription of a number of genes in response to the replacement of ammonia by leucine as source of nitrogen, including the general amino acid permease *GAP1* [27]. The microarays showed high hybridization spots for both genes *GLN3* and *GAP1* of cDNA from cells cultivated on L-leucine. The expression levels of other genes encoding transcription factors involved in nitrogen metabolism, *LEU3, SKP1* and *MET30,* were significantly reduced on L-leucine growth. There was no significant discrepancy in expression ratios in this group due to the difference in cell cultivation, batch or CSTR.

Cluster analysis of genome-wide and L-leucine specific expression patterns

We applied hierarchical clustering on the four datasets; a genome-wide clustering (Fig. 5A) and clustering on the data aggregation from the SAM-test (group of 117 genes, Fig. 5B), to depict biological order in the gene expression response. As is seen in Fig. 5A and 5B, in general the influence of cultivation methods on gene expression can be distinguished by a 'cultivation-dendrogram'. The dendrogram explicitly reveals the impact of the difference in cultivation method, batch or CSTR, above the difference in N-source. Thus genome-wide, the formation of flavour compounds makes little difference in the observed pattern of gene expression in comparison to a CSTR or batch cultivation. This result shows that gene profiles, compared across different fermentation conditions, do not contribute significantly to the identification of logical correlations. On the other hand, clustering of the group of 117 genes show a different dendrogram, resulting in the clustering of the two L-leucine cultivated cell experiments (Fig. 5B). Therefore clustering of the relatively small number of genes, obtained via non-identical conditions, demonstrates the value of the SAM-identified relevant genes, and strengthens the indication that these genes are particularly involved in flavour formation.

Transcriptional response of genes, specifically involved in flavour formation

Previously, the family of permeases was described, which is responsible for the uptake of L-leucine [28]. Yeast cells cultivated in the presence of L-leucine, genes encoding amino acid permeases or ammonium permease were affected during flavour forming conditions. The transcripts of *GAP1, BAP2, BAP3, ALP1, AGP2, TAT1* and *DIP5* were significantly upregulated during flavour formation in either batch or CSTR-cultured cells. The genes *BAP2, BAP3*s and *GAP1* contain UAS for G*LN3*.

Two branched chain transaminases encoded by *BAT1* and *BAT2,* are identified as enzymes responsible for the transaminase step in the Ehrlich pathway. Induced expression of *BAT1* was found under both culture conditions, whereas expression of *BAT2* was only observed in the L-leucine continuous culture cells. An explanation for undetected transcripts of *BAT2* in batch cultured cells can be that *BAT2* expression is growth rate dependent and induced during stationary growth phase, similar as *HXT5* [5, 34]. In addition, it has been described that *BAT2* has a minor role in cells, when cultivated on glucose whereas the presence of *BAT2* is essential during growth on ethanol [8]. Concerning the second step of the Ehrlich pathway, there are indications that pyruvate decarboxylases might be responsible for the decarboxylase step of the Ehrlich pathway. Expression patterns of genes encoding pyruvate decarboxylases and pyruvate decarboxylase-like proteins: *LPD1, PDA1, PDB1, PDC1, PDC5, PDC6* and *YDL080c* did not show a significant difference in gene expression during 3-methyl-1-butanol production. *PDX1*, pyruvate dehydrogenase complex protein X, the binding protein for Lpd1p, showed a significant increase in gene expression during flavour formation. This gene product plays an important role in the decarboxylation of branched chain oxoacids [29] and may catalyze the decarboxylation of 4-methyl-2-oxopentanoate to 3-methylbutyraldehyde. The last step of the Ehrlichpathway concerned a reduction step by via an alcoholdehydrogenase producing isoamyl alcohol out of the aldehyde [30]. Also none of the alcohol dehydrogenases showed a significantly altered gene activity. Transcripts of *YMR318c,* proposed to be involved in the synthesis of fusel alcohols [12], were present and could be clearly detected in the cell. However, the gene showed no significant change in gene activity. Another gene, *YCR105w*, also a NADPH-dependent alcohol dehydrogenase, with a 64% protein similarity to *YMR318c* was highly induced during leucine CSTR cultivation.

S. cerevisiae possesses two ORFs with similarity to human branched chain oxoacid dehydrogenase kinase, *YIL042c* and *YGL059w*. *YGL059w* showed a strong hybridization signal. The presence of a functional BC 2-oxoacid dehydrogenase complex in yeast can clarify the presence of metabolites like 3-methylbutyric acid via the formation of isovaleryl CoA. In its turn isovaleryl CoA could be further converted into isovaleric acid by an acetyl CoA hydrolase. The acetyl CoA hydrolase gene, *ACH1,* was significantly upregulated in CSTR cultivated cells, when grown on leucine. If the gene expression reflects proteins involved in this pathway, the presence of 3-methylbutyrate (isovaleric acid) can be explained by the existence of a functional BC oxoacid dehydrogenase complex.

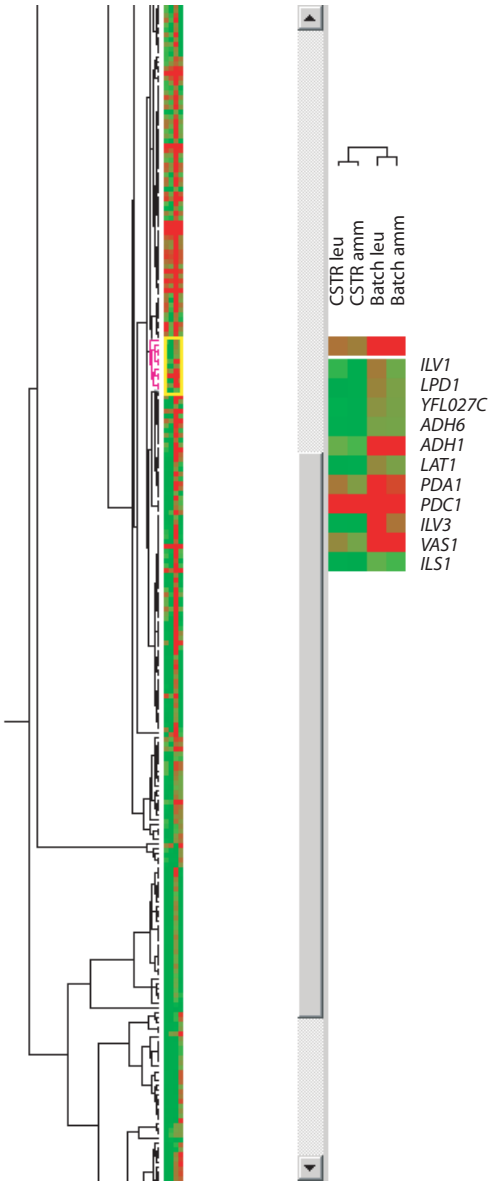

Figure 5A. Hierarchical clusters of the four datasets visualized by Treeview. Genome-wide clustering shows a cluster dendrogram for the datasets. The two datasets from samples from CSTR- and the two datasets from batch cultivation are clustered together, visualized by the horizontal dendrogram.

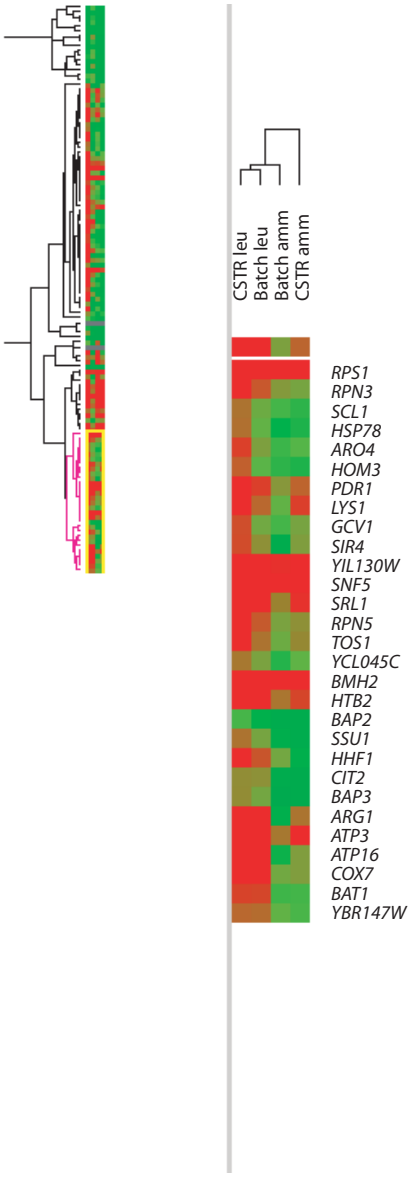

Figure 5B. Hierarchical clusters of the four datasets visualized by Treeview. Clustering on the specific group of 117 genes shows a dendrogram, clustering cells, cultivated on L-leucine together. CSTR-cultivated cells on ammonium sulphate displayed an expression pattern, apparently different to that of the batch cultivated cells.

Discussion

Flavour formation during food fermentation has been studied, since it was discovered that micro-organisms generated aroma substances during fermentation. High impact aroma molecules were derived from the conversion of different amino acids. Most enzymes involved in the metabolic pathway of these flavours have been identified biochemically [4]. However, the molecular mechanisms behind the production of flavour compounds have not been elcudated, yet. The conversion of branched chain amino acids into their respective oxoacids and alcohols was extensively studied via *BAT1* and *BAT2* deletion and over expression studies. The studies showed that the branched chain transaminases Bat1p and Bat2p catalyze the deamination of L-leucine into oxoacid, the first intermediates of the Ehrlich pathway [6, 5]. However, it has been proven that the branched chain amino acid transaminases were not essential for 3-methyl-1-butanol production by *S. cerevisiae*. Other following steps of the Ehrlich pathway were less extensively studied and prior to this research, no genes were identified to increase the production of the high impact flavour 3-methyl-1-butanol [31].

The aim of this study was to reinvestigate flavour formation by *S. cerevisiae*. Due to the development of cDNA microarrays, a tool is provided to identify expression of genes that function in metabolic pathways or as regulatory genes for the Ehrlich pathway.

We show that *S. cerevisiae* produced 4 volatile flavour substances when L-leucine was present in the media. Two of the emerged metabolites, 4-methyl-2-oxopentanoate and 3-methyl-1-butanol can directly be related to L-leucine catabolism, since deamination of L-leucine results in 4-methyl-2-oxopentanoate [29]. 3-Methyl-1-butanol is the final product of L-leucine degradation. The other detected products, 3-methyl-2-oxobutanoic acid is known as intermediary metabolite of the branched chain amino acid valine pathway and 3-methylbutyric acid is not known to be involved in any branched chain amino acid metabolism [9].

In this study, several genes were identified with altered expression levels when cells produced the volatile flavour products. Arrays from yeast cells harvested from two different cell cultivation methods, batch and CSTR, were also compared. Previous growth and biochemical studies with the soy yeast *Zygosaccharomyces rouxii* showed the interference of aspartate derived amino

acid metabolism when threonine was added to the medium [32]. Unsupervised analyses of the arrays exposed that during cultivation of both, batch and continuous growth conditions, a group of 117 genes were significantly altered when cells produced flavour compounds independently of the culturing method. These genes were involved in the amino acid metabolism of aromatic amino acids, serine, arginine, glutamine, homoserine and lycine, amine biosynthesis, caboxylic acid- and organic acid metabolism. Strikingly, genome-wide analysis of the L-leucine CSTR cells, L-leucine batch cells, ammonia CSTR cells and ammonia batch cells revealed a more similar transcription profile for CSTR cultivated cells or batch cultivated cells than for the L-leucine or ammonia cultivated cells. This was surpassed in the identified group of 117 genes, in which the transcription profiles of L-leucine or ammonia cultivated cells were clustered together. Therefore, these genes were identified as specific affected gene groups upon flavour formation deriving from L-leucine.

Furthermore, genes with regulatory elements for *GCN4* and *GLN3* were significantly present in the group of affected genes, identified during volatile flavour. Gcn4p, a basic leucine zipper protein, is identified as primary regulator of the transcriptional response to amino acid starvation [25]. It has been reported that Gcn4p is required for the full induction of at least 539 genes, involved in every amino acid biosynthetic pathway except cysteine and genes encoding amino acid precursors, vitamin biosynthetic enzymes, peroxisomal components, mitochondrial carrier proteins, and autophagy proteins [25]. Identification of Gcn4p, involved in flavour formation, which is closely related to the amino acid pathways is in agreement of previous transcriptional profiling results. The *GLN3* gene of *S. cerevisiae* is required for the activation of transcription of a number of genes in response to the replacement of glutamine by glutamate as source of nitrogen. Immunoprecipitation experiments indicated that the Gln3 protein binds the nitrogen upstream activation sequence of *GLN1*, the gene encoding glutamine synthetase [33]. Either the control of transcription or control of initiation of translation of *GLN3* and *GCN4* genes and target genes are important for the presence of volatile flavours in response to L-leucine presence.

Our results showed that in addition to previously identified genes *BAP2* and *BAP3*, which require Ssy1p-mediated signaling [28], *PDX1* and *YDL080c,* encoding decarboxylases for 4-methyl-2-oxopentanoate, *YMR318c* and *YCR105w*, encoding an alcohol dehydrogenase, genes homologous to leucine degradation genes in mammals and bacteria were upregulated in *S. cerevisiae*

during the flavour forming conditions. We discovered two genes with an increase in expression levels by cells producing fusel alcohols. These genes included *YGL059w,* an ORF with similarity to human branched chain oxoacid dehydrogenase kinase, and *ACH1*, an acetyl-CoA hydrolase. One of the genes *YGL059w* has been suggested to be involved in branched chain amino acid degradation. In addition, our data show that at least three genes *BAT1, PDX1* and *YCR105w* were significantly altered when cells produced flavour substances and independently of culture conditions.

Therefore, transcriptional activation of genes, involved in flavour formation, shows connection of apparently unconnected pathways: carboxylic acid or other amino acid metabolisms. In addition to the well known Ehrlich pathways for fusel alcohol production [3] cDNA microarray data indicate that the formation of the detected volatile flavours, 3-methyl-1-butanol, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid, may occur through an oxidative decarboxylated manner, very similar to mammalian cells (Fig. 6).

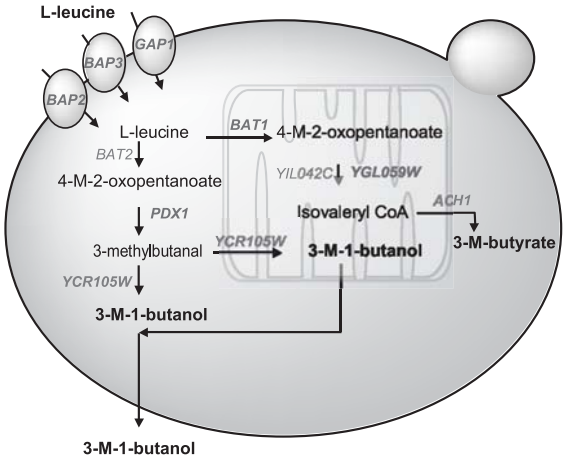

Figure 6. Schematic representation of metabolic routes of L-leucine, leading to 4-methyl-2-oxopentanoate, 3-methyl-1-butanol and 3-methylbutyrate. Genes, enhanced in expression in one of the investigated condition are depicted. Genes, enhanced in expression under batch and CSTR culturing conditions are depicted in bold.

It became clear that array data cannot simply be compared with other array data. We show that cell cultivation methods, regardless of the N-metabolism, have major influence on the transcriptome. In our case we identified group of genes with an expression profile correlated with the formation of aroma compounds; further identification of the enzymes and genes involved may ultimately enable the optimization and control of a desired blend of flavours.

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CHAPTER FIVE

Rapid identification of target genes for 1-methyl-3-butanol production in *Saccharomyces cerevisiae*

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Abstract

Extracellular conditions determine the taste of fermented foods by affecting metabolite formation by the micro-organisms involved. To identify targets for improvement of metabolite formation in food fermentation processes, automated high-throughput screening and cDNA microarray approaches were applied. *Saccharomyces cerevisiae* was cultivated in 96 wells microtiter plates and the effects of salt-concentration and pH on growth and synthesis of the fusel alcohol flavour substance, 3-methyl-1-butanol, was evaluated. Optimal fermentation conditions for 3-methyl-1-butanol concentration were found at pH 3.0 and 0% NaCl. To identify genes encoding enzymes with major influence on product formation, a genome-wide gene expression analyses was carried out with *S. cerevisiae* cells grown at pH 3.0 (optimal for 3-methyl-1-butanol formation) and pH 5.0 (yeast cultivated under standard conditions). A subset of 747 genes was significantly induced or repressed when the pH was changed from pH 5.0 to pH 3.0. Expression of 7 genes related to the 3-methyl-1-butanol pathway, *LAT1, PDX1, THI3, ALD4, ILV3, ILV5* and *LEU4* strongly changed in response to this switch in pH of the growth medium. In addition, genes involved in NAD-metabolism, *BNA2, BNA3, BNA4,* and *BNA6,* or involved in the TCA-cycle and glutamate metabolism, *MEU1, CIT1, CIT2, KDG1* and *KDG2,* displayed significant changes in expression. The results indicate that this is a rapid and valuable approach for identification of interesting target genes for improvement of yeast strains used in industrial processes.

Introduction

Food fermentation covers a wide range of microbial processing aiming to achieve desirable product characteristics such as prolonged shelf life, nutritional enrichment and attractive flavour. Changes in extracellular conditions during food fermentation have great impact on the final product quality. They influence metabolic fluxes, affecting cell growth and the production of flavour compounds [1, 2]. Important issues in industrial food fermentation concern the efficiency of the process; i.e. quality, taste and process time. Improvements in efficiency are usually obtained via strain selection or by strain improvement [3]. Consequently, up-to-date knowledge is required of the physiology, metabolism and genetic properties of the fermenting micro-organisms.

High-throughput techniques for metabolite screening and gene expression analysis provide suitable tools for detailed analysis of microbial physiology. We applied a powerful combination of a microtiter plate (MTP) metabolite screening assay with high-density cDNA filters (microarray gene expression analysis) to investigate the production of fusel alcohols. These alcohols are important flavour substances in fermented foods. Since in many food fermentations, such as soy sauce, a pH and salt-step is included, we investigate effects of changes in important extracellular parameters such as pH and salt-concentration on formation of fusel alcohols in *S. cerevisiae*.

The MTP metabolite screening assay allows rapid screening of metabolite formation under different fermentation conditions. Cells are cultivated in 96 deep well MTPs, each well representing an individual extracellular condition with minor variations in pH and salt concentration. Using a dedicated facility with several MTP handling and pipetting robots (http://www.bioexplore.com), large numbers of conditions can be screened in a relatively short time. The compounds of interest produced, e.g. amino acids and alcohols can be analyzed using GC, or HPLC-MS methods [16].

cDNA microarrays visualize the genome-wide expression profile of an organism. New sets of induced and repressed genes have been identified by the use of microarrays, in the response to gene deletions or to various extracellular changes [4]. Using optimal growth conditions for metabolite formation, established by the high-throughput metabolite screening, cDNA microarray analysis was applied to identify metabolic processes and pathways involved in

fusel alcohol production in *S. cerevisiae*. The latter are potential targets for subsequent strain improvement studies.

Fusel alcohols, 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-butanol (active amyl alcohol) and 2-methyl-1-propanol (isobutyl alcohol), are produced during the catabolism of branched chain amino acids (BCAA; leucine, isoleucine and valine) [5] or they are synthesized *de novo*, during the biosynthesis of the BCAA [6]. However, the precise pathways for fusel alcohol production by yeast are poorly understood at present. The effects of important food fermentation parameters, such as pH and salt-concentration on underlying molecular mechanisms remain unknown.

The aim of this study is to identify genes in *S. cerevisiae*, leading to the secondary metabolite 3-methyl-1-butanol in conditions relevant to food fermentation. The metabolite screening exposed the effects of pH and NaCl on the fermentative pattern of *S. cerevisiae*: optimal growth conditions for fusel alcohol synthesis were pH 3.0 and 0% NaCl. The gene expression profiles showed an upregulation of specific genes, encoding for enzymes involved in fusel alcohol production at low pH. Also, genes involved in leucine metabolism and NAD-metabolism, were strongly expressed under these conditions.

Materials and methods

Strain, growth media and cultivation procedures

Saccharomyces cerevisiae wild type strain CEN.PK113-7D was used. Cultures were maintained on skimmed milk and glycerol at –80^{0}C. Precultering of *S. cerevisiae* was done at 30^{0}C and 150 rpm on a rotary shaker (Unitron AJ200 shaker, Infors AG, Bottmingen, Switzerland), in 500 ml erlenmeyer flasks containing 100 ml medium. The medium used was yeast nitrogen base (YNB) without ammonium sulphate and amino acids (Difco Laboratories, MI, USA), supplemented with 2% glucose (autoclaved), $(NH_4)_2SO_4$ (5 mM) and citrate buffer (0.2 M; also containing 0.1 M KH_2PO_4) at pH 5.0. The complete medium was filter sterilized (Millex 0.22 μm, Millipore, Billerica, MA, USA). Cultures were grown until exhausted of the nitrogen source and cells were stored at 4^{0}C and used as inoculum for further experiments. Experiments in batch cultures (0.5% inoculum) were done in duplicate as described above, with L-leucine (10 mM) as nitrogen source at 30^{o}C and 150 rpm. At different time points optical density at 600 nm and 3-methyl-1-butanol concentrations were measured.

Adaptation to high NaCl concentrations was achieved by transferring cultures (1% v/v) to fresh medium containing 2% NaCl and grown until exhausted of nitrogen source. This procedure was repeated three times, increasing NaCl concentration with 2% per step, until reaching 8% NaCl. Experiments in deep-well (96-well) microtiter plates with a pH gradient (in one direction at 8 different values of pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0) and a NaCl gradient (in the other direction with 6 different concentrations of 0 to 10% NaCl with a step-size of 2% NaCl) were inoculated to an OD_{600} of 1.0, and incubated at 30 ºC. This high initial OD_{600} value was chosen in order to reduce differences in lag-phase between different wells. The inocula for wells with medium containing 0% NaCl/ 2% NaCl/ 4% NaCl/ 6% NaCl/ 8 and 10% NaCl were precultured on medium containing 0%/2%/4%/6%/8% NaCl, respectively. Erlenmeyer flasks (500 ml, containing 100 ml medium) were incubated at 150 rpm on a rotary shaker, and (deep-well) microtiter plates. The microtiter plates were capped to prevent evaporation (Fig. 1). MTPs were shaken at 900 rpm (total orbit of 1.5 mm) on a Titramax 1000 rotary shaker (Heidolph Instruments, Schwabach, Germany). After rapid cooling of microtiter plates, or samples, at 4^0C for at least 30 min, metabolites were measured in supernatants obtained after centrifugation using a centrifuge with a rotor for (deep-well) microtiter plates (Sigma 4-15C, Sigma laboratory centrifuges GmbH, Osterode, Germany).

Figure 1. 96-deepwell MTP, used for scale down fermentations to establish the effects of salinity stress and pH, on growth and formed metabolites. The MTP was sealed with an accurate fitted silicon cap, to prevent evaporation of volatile compounds.

Analytical methods

3-Methyl-1-butanol concentrations were determined by gas chromatography with flame ionization detector (GC 8000 Top equipped with two autosamplers in order to double the number of samples that can be handled at the same time; CE Instruments, Thermoquest, Milan, Italy), using on-column injection and He as carrier gas. Columns used were Zebron ZB-1 (30 m, ID 0.32 mm, 0.50 μm film, Phenomenex, Torrance, CA, USA, and DB-WAX (30 m, ID 0.53 mm, 1.00 μm film, J and W Scientific, Folsom, CA, USA). Samples were extracted with methyl formate (1:3), containing 1-hexanol or 1-pentanol as internal standards.

Methods were optimized for fast separation by using short temperature gradients to increase the throughput of samples (retention times of 5 min or less). L-leucine was measured using HPLC after derivatization with 30 µl *o*-phthalaldehyde/3-mercaptopropionate mixture (25 mM *o*-phthalaldehyde and 25 mM 3-mercaptopropionate in 50% methanol) and 200 µl 0.3 M borate buffer (pH 9.3). The system was equipped with a Luna 5 µC18 column (30×4.60 mm, Phenomenex) and a UV6000LP diode array detector (Thermo Separation Products, San Jose, CA, USA); the eluate was monitored at 335 nm.

Growth was measured in a polycarbonate microtiter plate at 600 nm after appropriate dilution in water, using a spectrophotometer (SPECTRAmax PLUS384 Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA, USA). Above-mentioned assays were routinely done using automated robotized equipment for pipetting and plate handling: the Plato-7 pipetting robot (Rosys-Anthos, Hombrechtikon, Switzerland), the Zymate robot for plate handling, and the RapidPlate-96 pipetting robot (Zymark, MA, USA). Contour plots were made using MATLAB version 6.1.0.450 Release 12.1 (MathWorks, Natick, MA, USA).

Cell extract preparation, synthesis of cDNA probe, GeneFilter hybridization, data analysis and spot validation

Samples for cell extracts were taken from the erlemeyer cultures at t = 24 h. Cell extracts and [^{33}P]CTP-labelled cDNA were prepared as previously described [7]. The procedure for hybridization of the GeneFilter membranes (Life Technologies, Breda, The Netherlands), data analysis (using ImaGene 4.0 microarray analysis software, BioDiscovery, Marina del Rey, CA, USA) and spot validation was performed as described in Schoondermark-Stolk et al., 2002 [30]. The expression profile of *S. cerevisiae* cells cultivated at pH 3.0 and pH 5.0 were investigated with two independent duplo microarray hybridizations. After scanning and image processing the filters were stripped and hybridized a second time with probes from the same batch. In this way, 4 genome-wide datasets were obtained for pH 3.0 as well as for pH 5.0.

Data normalisation was performed by the web tool SNOMAD (Standadization and NOrmalization of MicroArray Data) [8]. By using SNOMAD, the influence of artefacts of mean intensities locally across the surface of the DNA microarray was reduced (Fig. 2A, B). Normalization was executed for each pH 5.0 - pH 3.0 set. After SNOMAD normalisation, the 4 sets were normalized on total spot intensities. The program SAM: Significance Analysis of Microarrays, was used

for statistical support to determine if the expression of any genes were significantly related to the difference in pH (2-class responses, [9]), by comparing the difference between the quadruplicate measurements to the difference between the pH 3.0 and pH 5.0 datasets.

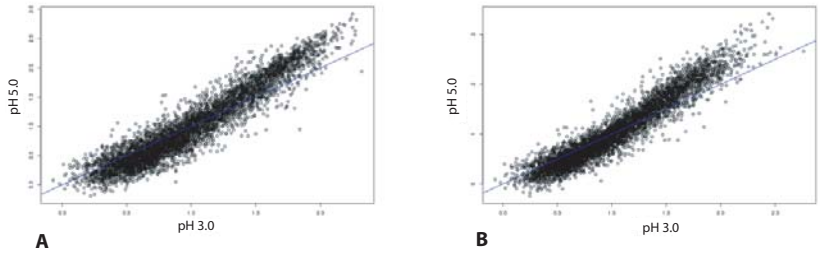

Figure 2A-B. Visualisation of all spot intensities of pH 5.0 and pH 3.0 cells obtained by the cDNA microarray. Values are expressed in arbitrary units (A.U.). There are no significant differences between A and B, indicating high quality and equally hybridized arrays. High intensity spots (A.U. >1.5) were observed for control cells, pH 5.0. Low and moderate intensity spots (0<A.U.<1.5) were equally present in pH 3.0 and control cells. Scatterplot of raw microarray data (A). Scatterplot of SNOMAD normalized data. Variation in low intensity spots (A.U.<1) was minimized according to SNOMAD algoritm (B).

Results

Automated high-throughput assay of yeast cell growth in deep-well microtiter plates

Growth performance and fermentation processes of the yeast *S. cerevisiae,* have been studied intensively, using solid-state agar plates, shake flasks or (large-scale) fermentors. Recently, it has been reported that also MTPs can also be used to cultivate yeast in suspension [10, 11]. We have utilized sealed round-bottom deep-well MTPs, to identify optimal pH values and NaCl-concentrations for fusel alcohol formation by *S. cerevisiae*. Each well, with a total volume of 1200 µl, represented a microculture in 300 µl medium. The incubations were performed at 30°C on YNB medium with L-leucine as N-source and pH and NaCl gradients were applied as described in materials and methods. The optical density, L-leucine and 3-methyl-1-butanol concentrations of the microcultures were measured after 140 h of culturing. A change in pH over the range 3.0-7.0 had no major influence on the final OD_{600} of the yeast after 140 h culturing,

when no NaCl was present in the medium. Changes in pH in combination with NaCl on the other hand, caused significantly reduced growth at pH < 4.0 with NaCl-concentrations of 2.5% w/v and higher (Fig. 3A). In addition, in an acidic environment at pH below 4.0, elevated 3-methyl-1-butanol concentrations were measured (Fig. 3C). A maximal formation of 3.4 mM 3-methyl-1-butanol was established at pH 3.0. A clear correlation was observed between growth (Fig. 3A) and the external L-leucine concentration (Fig. 3B). When the cells reached a maximum OD_{600} of 15, no residual L-leucine was measured in the medium, indicating that all L-leucine had been consumed. No residual L-Leucine was detected when the extracellular NaCl-concentration remained under 6% w/v. NaCl had an inhibitory effect on the 3-methyl-1-butanol formation (Fig. 3C). Thus, MTP screening revealed that *S. cerevisiae* formed the highest 3-methyl-1-butanol concentration (3.4 mM) at low pH (pH 3.0) in absence of NaCl (Fig. 3C).

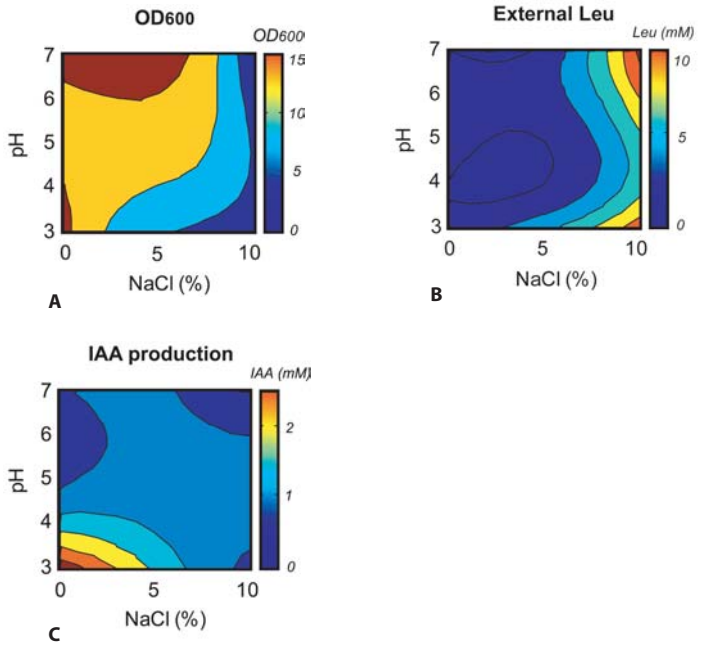

Figure 3A–C. Contour plots of *S. cerevisiae* grown in deep-well microtiter plates in medium with 6% glucose and 10 mM leucine at 30 ºC with final values of OD600 (A), L-leucine (B, mM),), 3-methyl-1-butanol (C, mM). Vertical bars indicate OD600 values or metabolite concen-trations (see colour version at page 138).

Gene expression analysis

To identify candidate genes involved in fusel alcohol formation, whose expression is affected by a lowering of extracellular pH, we compared the genome-wide gene expression profiles of *S. cerevisiae* grown at pH 3.0 with pH 5.0, the standard cultivation pH, without any NaCl present. Yeast growth and product formation in the MTP cultures can be extrapolated to Erlenmeyer flask batch cultures [10]. In 500 ml Erlenmeyer flasks under similar conditions as MTP cultures, the 3-methyl-1-butanol concentration reached a maximum of 5.4 mM, with a yield of 0.74 mmol 3-methyl-1-butanol per OD_{600} at pH 5.0 (Fig. 4A). At pH 3.0 the 3-methyl-1-butanol yield increased with 13% compared to the control, and reached 0.85 mmol per OD_{600} (Fig. 4B). Although the difference in 3-methyl-1-butanol yield was less obvious as observed in the MTP, cells cultivated at pH 3.0 showed the highest flavour production.

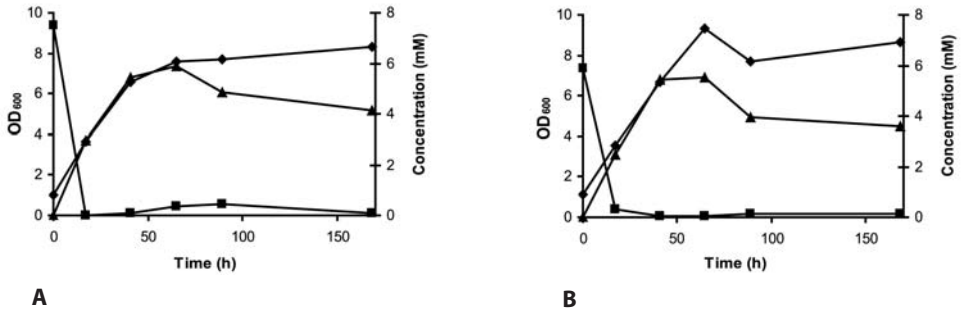

Figure 4A–B. Growth curves of *S. cerevisiae* in Erlenmeyer flasks in medium with 6% glucose and 10 mM leucine at 30 ºC. Growth conditions are pH 5.0 (A) and pH 3.0 (B). Symbols: ◆, OD600 (left axis); ■, leucine concentration (mM); ▲, 3-methyl-1-butanol concentration (mM).

Total cellular RNAs were isolated from the cells and used as templates for ^{33}P-labeled cDNAs. ^{33}P-Labeled cDNAs were then hybridized with the DNA filters, containing 6144 ORFs, covering >95% of the total ORFs of the *S. cerevisiae* genome database. Using the software program Significant Analysis of Microarrays (SAM) [9] and a fold-change threshold >2, 747 genes were identified with an altered expression level in pH 3.0 cells as compared to the control. A total of 388 genes showed a down regulation at pH 3.0 and 359 genes were up regulated at pH 3.0.

Characterization of expression of genes involved in fusel alcohol production.

Cellular fusel alcohol production is the net result of activities of both, the BCAA degradation and biosynthesis pathways. Expression ratios of 13 genes were established, encoding enzymes involved in these pathways according to the Kegg Pathway Database [12, 13], (Table 1). Three genes, involved in the L-leucine degradation, *BAT1, BAT2* and *ERG13,* encoding a mitochondrial branched chain amino acid transferase, cytosolic branched chain amino acid transferase and 3-hydroxy-3-methylglutaryl-CoA synthase, respectively, were clearly expressed (Fig. 5). The genes *LEU1* and *LEU2,* involved in the leucine biosynthesis, encoding 3-isopropylmalate dehydratase and 3-isopropylmalate dehydrogenase, respectively, were also clearly expressed (Fig. 5). *ALD6* (cytosolic aldehyde dehydrogenase), *ILS1* (isoleucine-tRNA ligase), *ILV2* (acetolactate synthase), and VAS1 (valyl-tRNA synthase), involved in the isoleucine and valine pathways, showed high transcriptional activities. Expression of these genes was observed at both, pH 5.0 and pH 3.0. The genes *ALD4* (mitochondrial aldehyde dehydrogenase), *ILV3* (mitochondrial dihydroxy-acid dehydratase), *ILV5* (mitochondrial ketol-acid reductoisomerase) and *LEU4* (mitochondrial 2-isopropylmalate synthase), were only expressed in cells cultivated at pH 5.0. No expression of *HAT2* (subunit of the major yeast histone acetyltransferase), *HPA3* (histone and other protein acetyltransferase), *CDC60* (leucine-tRNA ligase), *ILV6* (regulatory subunit of acetolactate synthase), *ISM1* (isoleucine-tRNA ligase), *NAM2* (leucyl-tRNA synthase), *MAK3* (N-acetyltransferase), *POT1* (peroxisomal acetyl-CoAC-acyltransferase) and *ALD5* (mitochondrial aldehyde dehydrogenase (NAD+)), was detected neither in pH 3.0 nor in pH 5.0 grown cells.

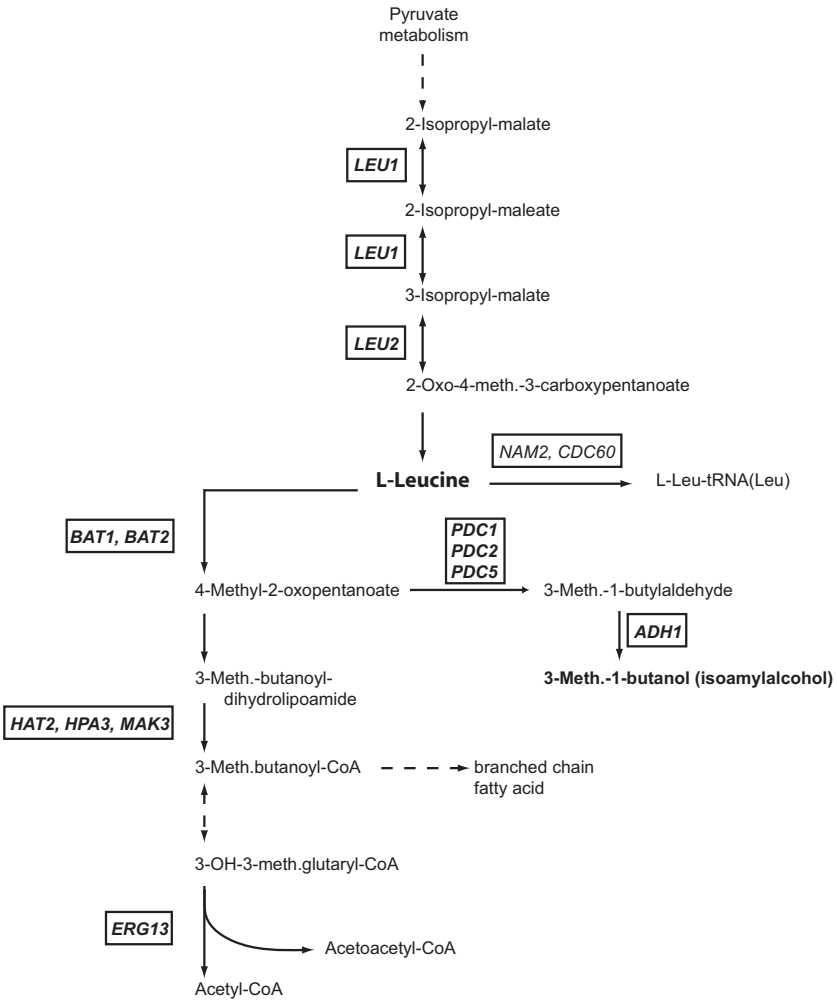

Figure 5. Representation of the leucine, valine, isoleucine biosynthesis and degradation route, ORFs, coding for either transcription factors or enzymes are included in the scheme according the KEGG database. Bold text reflects upregulation at pH 3.0, normal text reflects gene activity at pH 3.0 and in the control.

Table 1. Hybridization signals referring to enzymes involved in the metabolic pathway for valine, leucine and isoleucine, according to the Kegg Pathway Database [12]. Normalized spot intensity values in columns pH 5.0 and pH 3.0 are given in A.U. 2-fold up or down regulation is calculated in the column ^{2}log (pH 3.0 ·(pH 5.0)$^{-1}$)

Gene ID	pH5	pH 3	2log (pH 3 / pH 5)	biological_process
ALD4	24.6	-	-14.59	ethanol metabolism
ALD6	23.8	14.4	-0.73	carbohydrate metabolism
BAT1	13.6	18.1	0.41	branched chain family amino acid metabolism
BAT2	4.09	3.57	-0.20	branched chain family amino acid metabolism
ERG13	26.6	20.3	-0.39	ergosterol biosynthesis
ILS1	6.06	4.62	-0.39	isoleucyl-tRNA aminoacylation
ILV2	6.60	5.34	-0.30	branched chain family amino acid biosynthesis
ILV3	9.61	-	-13.23	valine isoleucine biosynthesis
ILV5	6.61	-	-12.69	not yet annotated
LEU1	17.1	17.8	0.05	leucine biosynthesis
LEU2	27.1	17.2	-0.66	leucine biosynthesis
LEU4	4.38	-	-12.10	leucine biosynthesis
VAS1	4.01	1.63	-1.30	valyl-tRNA aminoacylation

*For calculating ^{2}log ratio, missing value was replaced by an ignorable value of 0.001

In addition to the genes mentioned above, the ratios for another 11 genes, linked to fusel alcohol production as described in the literature, was established (Table 2). Among these genes are permeases, decarboxylases and dehydrogenases. Transcripts of *BAP2* and *BAP3,* encoding for a high-affinity leucine permease and valine transporter, were only detected at pH 3.0 (Table 2). The genes *GAP1* (general amino acid permease), *ADH1* (cytosolic alcohol dehydrogenase), *PDC1* (pyruvate decarboxylase isozyme 1), *PDC5* (pyruvate decarboxylase isozyme 2), *PDC6* (pyruvate decarboxylase isozyme 3) , and *YAL061W* (member of alcohol dehydrogenase family) were expressed at both pH values, without significant differences. Another 3 genes, *LAT1* (part of pyruvate dehydrogenase complex), *PDX1* (pyruvate dehydrogenase complex protein X), and *THI3* (4-methyl-2-oxopentanoic acid decarboxylase), were down regulated at pH 3.0. Transcripts of regulators like *LEU3* and *SSY1*, encoding for a transcriptional regulator of several transporter genes including *BAP2* [14], were not detected. Transcripts of *GCN4,* general regulator of gene expression during amino acid starvation [15], were detected at both, pH 3.0 and pH 5.0.

Of the gene groups involved in fusel alcohol production, a total of 17 genes were strongly expressed at pH 3.0. Nine of them, *BAP2, BAP3, LAT1, PDX1, THI3, ALD4, ILV3, ILV5* and *LEU4* displayed an altered gene expression. These genes may play critical roles in the enhanced fusel alcohol production at pH 3.0.

Table 2. Hybridization signals referring to enzymes involved in the metabolic pathway for valine, leucine and isoleucine, according to the literature. Normalized spot intensity values in columns pH 5.0 and pH 3.0 are given in A.U. 2-fold up or down regulation is calculated in the column ^{2}log (pH 3.0 ·(pH 5.0)$^{-1}$)

Gene ID	pH5	pH 3	2log(pH 3 / pH 5)	biological_process	ref
ADH1	15.8	16.2	0.04	carbohydrate metabolism	[23]
BAP2	-	3.34	11.7	amino acid transport	[24], [25], [26]
BAP3	-	1.90	10.9	amino acid transport	[27]
GAP1	152	87.3	-0.80	amino acid transport	[28]
LAT1	7.12	-	-12.8	not yet annotated	[29],
PDC1	309	332	0.10	ethanol fermentation	[22], [30]
PDC5	11.8	7.01	-0.75	ethanol fermentation	[22], [31]
PDC6	8.76	12.2	0.48	pyruvate metabolism	[23]
PDX1	2.92	-	-11.5	glycolysis	[32]
THI3	7.33	-	-12.8	thiamin biosynthesis	[18]
YAL061W	11.9	12.9	0.12	metabolism	[23]

* For calculating ^{2}log ratio, missing value was replaced by an ignorable value of 0.001

Characterization of expression of gene groups, linked to fusel alcohol production.

Using over-represented gene ontology (GO) groups of up regulated genes, alternative potential target genes for strain improvement were found. From the genome wide profile, we identified 28 different gene ontology (GO) groups, significantly up regulated at pH 3.0 (Table 3) [16]. From the 359 up regulated genes at pH 3.0, 358 were annotated to a given GO group (directly or indirectly). Since a gene may appear in several GO groups, the probability of having x out of n annotations assigned to the same GO group by chance, was defined as the product of the number of permutations by which the annotations could occur. In order to calculate the probability of 358 (x) of the 359 (n) genes to be annotated to a particular group, the following formulae was used by SGD Gene Term Finder software:

$$\sum_{j=x}^{n} \left(\frac{n}{j!(n-j)!} \right) \times p \times (1-p)^{(n-j)}$$

We applied a cut-off of $p<0.01$.

Table 3. Set of yeast genes that are up regulated at pH 3.0, categorized in Gene Ontology (GO) groups, based on similarity in molecular function, biological process and cellular component of gene products.

GO_term	Gene(s)
NAD biosynthesis	*BNA6 BNA2 BNA4 BNA3*
pyridine nucleotide biosynthesis	*BNA6 BNA2 BNA4 BNA3*
nicotinamide metabolism	*YOR356W BNA6 BNA2 RPE1 BNA4 MDH3 BNA3*
transport	*GBP2 VPS8 SNC1 FUR4 PMA2 PDS1 DNF1 NUP49 ECM1 HUT1 ODC1 STE6 INP54 SRP14 ODC2 CHS7 AVT7 ERP4 PDR12 FIT3 TPN1 MPH2 CHC1 SUL2 KAP120 SFB2 ERV14 NUP157 VPS68 FEN1 GSP1 GGC1 PEP1 VPS54 VMA8 DRS2 AVT5 ANT1 ATG12 SYN8 ERV41 BOS1 VHT1 FET5 SCO2 SAR1 YIP3 PDR16 ATP17 AVT6 COG5 YBT1 FIT2 DAL4 FTR1 KHA1 MUP3 YHC3 COT1 VAC8 FET3 SYS1*
NAD metabolism	*BNA6 BNA2 BNA4 BNA3*
pyridine nucleotide metabolism	*YOR356W BNA6 BNA2 RPE1 BNA4 MDH3 BNA3*
coenzyme and prosthetic group metabolism	*HEM2 MET8 ECM38 ISU2 YOR356W BNA6 BNA2 RPE1 BNA4 YAH1 DFR1 MDH3 ATP17 BIO4 BNA3*
water-soluble vitamin biosynthesis	*RIB5 THI13 BNA6 BNA2 BNA4 BIO4 BNA3*
vitamin biosynthesis	*RIB5 THI13 BNA6 BNA2 BNA4 BIO4 BNA3*
water-soluble vitamin metabolism	*RIB5 THI13 YOR356W BNA6 BNA2 RPE1 BNA4 MDH3 BIO4 BNA3*
vitamin metabolism	*RIB5 THI13 YOR356W BNA6 BNA2 RPE1 BNA4 MDH3 BIO4 BNA3*
fatty acid metabolism	*OAF1 SPS19 YBR159W FEN1 ANT1 MDH3 FAS2*
glutamate biosynthesis	*MEU1 CIT2 CIT1 PUT2*
coenzyme catabolism	*ECM38 YOR356W*
lipid biosynthesis	*GPI11 IDI1 YBR159W GWT1 MVD1 FEN1 FAS2 DGA1 PDR16 LAC1 GPI16 ERG10 CHO2*
organic acid metabolism	*ARO4 OAF1 SPS19 MEU1 HOM2 PDR12 TRP1 YBR159W CIT2 CYS4 FEN1 CYS3 TRP4 ANT1 DFR1 MDH3 CIT1 FAS2 MET22 PUT2 BIO4 SER1 YBL036C*
carboxylic acid metabolism	*ARO4 OAF1 SPS19 MEU1 HOM2 PDR12 TRP1 YBR159W CIT2 CYS4 FEN1 CYS3 TRP4 ANT1 DFR1 MDH3 CIT1 FAS2 MET22 PUT2 BIO4 SER1 YBL036C*
galactose metabolism	*GAL1 GAL80 GAL10*
oxidoreduction coenzyme metabolism	*YOR356W BNA6 BNA2 RPE1 BNA4 MDH3 BNA3*
heterocycle metabolism	*HEM2 AAH1 MET8 TRP1 TRP4 YAH1 DFR1 BIO4 SER1*
glutamate metabolism	*MEU1 CIT2 CIT1 PUT2*
mitotic spindle elongation	*APC2 APC9 APC1 UBC9*
lipid metabolism	*OAF1 SPS19 GPI11 IDI1 YBR159W YPC1 GWT1 MVD1 FEN1 ANT1 MDH3 FAS2 DGA1 PDR16 LAC1 GPI16 ERG10 CHO2*
coenzyme metabolism	*ECM38 ISU2 YOR356W BNA6 BNA2 RPE1 BNA4 DFR1 MDH3 ATP17 BIO4 BNA3*
nucleotide transport	*GGC1 ANT1*
coenzyme and prosthetic group catabolism	*ECM38 YOR356W*
nucleobase transport	*FUR4 DAL4*
purine nucleotide transport	*GGC1 ANT1*

Significant functional categories of the 359 genes, specifically enhanced during growth at pH 3.0, were linked to NAD pathways (NAD biosynthesis (p=0.00024), pyridine nucleotide biosynthesis (p=0.00044), nicotinamide metabolism (p=0.00068), and NAD metabolism (p=0.00112)) and transport (p=0.00075). NAD is a coenzyme involved in oxidoreductase reactions, amongst them the conversion of branched chain aldehydes to fusel alcohols. *BNA6, BNA2, BNA4* and *BNA3,* encoding enzymes required for biosynthesis of nicotinic acid from tryptophan via the kynurenine pathway, were significantly up regulated at pH 3.0. *YOR356W, RPE1* and *MDH3,* three other predicted genes in the nicotinamide pathway, including NAD and NADP, were also up regulated at pH 3.0. In addition to the groups associated with NAD also the group of vitamin biosynthesis was extracted as significantly enhanced at pH 3.0. The following GO identified groups concerned coenzyme and prosthetic group metabolism (p=0.00190), vitamin biosynthesis (p=0.00231), water-soluble vitamin biosynthesis (p=0.00231), vitamin metabolism (p=0.00316) and water-soluble vitamin metabolism (p=0.00316).

The remaining GO groups identified, included the group of glutamate biosynthesis and carboxylic acid metabolism. Four out of 13 annotated genes are involved in the glutamate biosynthesis, *MEU1* (s-methyl-5-thioadenosine phosphorylase), *CIT2* (peroxisomal citrate synthase), *CIT1* (mitochondrial citrate-synthase), and *PUT2* (1-pyrroline-5-carboxylate dehydrogenase) were significantly up regulated at pH 3.0. Glutamate is formed from 2-oxoglutarate, derived from the TCA cycle. The activity of genes encoding for enzymes responsible for the conversion of 2-oxoglutarate to succinate, *KDG1* and *KDG2,* was reduced at pH 3.0 with a ^{2}log pH-ratio of -0.15 and –1.03, respectively. The transamination of the nitrogen group of leucine to 2-oxoglutarate yields 4-methyl-2-oxopentanoate and glutamate. In turn 4-methyl-2-oxopentanoate is the precursor of 3-methyl-1-butanol (Fig. 6). Thus, not only genes directly involved in the branched chain amino acid metabolism, also genes involved in energy generation and genes involved in other related pathways, such as the TCA cycle and glutamate biosynthesis, were identified as genes of interest, putatively stimulating 3-methyl-1-butanol production.

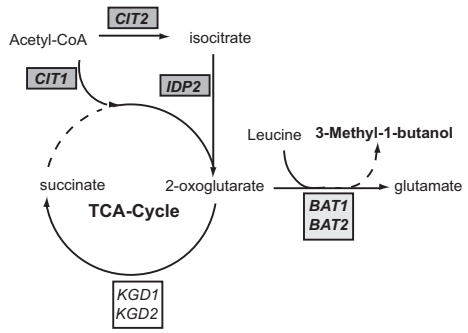

Figure 6. Schematic representation of pathways related to 3-methyl-1-butanol production. 2-Oxoglutarate is converted to succinate the TCA cycle via Kdg1 and Kdg2. The activity of genes encoding for the enzymes was reduced at pH 3.0. Genes encoding for Cit1, Cit2 and Idp2 were upregulated at pH 3.0

Discussion

We have designed a two-factor combinatorial assay for a rapid and reliable identification of optimal fermentation conditions, and elucidation of genes, encoding enzymes with major influence on product formation. The effects of two important food fermentation process parameters, pH and NaCl, were tested. The fusel alcohol, 3-methyl-1-butanol, was used as indicator substance, relevant for the taste of fermented foods. As shown in Fig. 3A-C, changes in cell growth, L-leucine consumption and 3-methyl-1-butanol production, are visualized by the automated high-throughput MTP screening. The data show that the presence of NaCl had an inhibitory effect on the fusel alcohol formation in *S. cerevisiae*. An acidic environment below pH 5.0, amplified the inhibitory effect of NaCl on fusel alcohol production by the yeast. Furthermore, the cells did not exhibit growth- or L-leucine consumption-dependent responses for fusel alcohol formation. Thus, no direct relation between the uptake of branched chain amino acids (L-leucine) and the formation of branched chain alcohols (3-methyl-1-butanol) was observed. The highest fusel alcohol formation was measured at pH 3.0-4.0 without NaCl, associated with moderate L-leucine consumption and moderate growth. This screening assay already has allowed identification of similar extracellular parameters, crucial for fusel alcohol formation in soy sauce, by the yeast *Zygosaccharomyces rouxii:* highest fusel alcohol concentrations were obtained at low pH (3.0-4.0) and low NaCl-concentration (0-4%) [10].

Fusel alcohol production is a major topic in food and beverage fermentations, several studies have been performed to elucidate bottlenecks in the fusel alcohol formation pathway. Disruption or over expression of obvious target genes in *S. cerevisiae*, such as the BCAA transporters *BAP2, BAP3,* the BCAA transferases, *BAT1* and *BAT2* and 4 pyruvate decarboxylases, *PDC1*, *PDC5*, *PDC6* and *YDL080c*, showed that neither the uptake, nor the transamination or the decarboxylation of the BCAA is essential for the formation of fusel alcohols, [17, 18, 19, 20, 21, 22]. These observations emphasized the necessity for further research on fusel alcohol production. Microarray technology offers a possibility to identify genes that play an important role in the formation of fusel alcohols, allowing directed strain improvement in further work.

The optimal fermentation condition for fusel alcohol production was used for cDNA microarray studies. The cDNA microarray studies pointed to 747 genes with a significantly altered transcription activity. The different metabolic pathways and processes responsible for fusel alcohol production thus may be identified from these changed gene expression levels. The cDNA microarray analysis demonstrated that 17 genes, encoding steps in BCAA metabolism, were strongly expressed in the yeast cells. A subset of 9 genes, *BAP2, BAP3, LAT1, PDX1, THI3, ALD4, ILV3, ILV5* and *LEU4,* showed a significantly changed gene expression level at pH 3.0 compared to the standard pH 5.0. It has been reported that the uptake of BCAAs is not the bottleneck in fusel alcohol production [17, 21], which leaves the last 7 genes as targets for strain improvement.

In addition to these target genes that may contribute to fusel alcohol production, we observed that numerous nicotinamide and vitamin biosynthetic genes were induced during growth at pH 3.0. This was revealed by SGD Gene Ontology (GO) Term Finder. Identified groups included the metabolism of nicotinamides, vitamins, fatty acids, glutamate and carboxylic acids (Table 3). Vitamins function for instance, as cofactors for various enzymes involved in metabolic pathways. Up regulation of genes involved in nicotinamide biosynthesis may be explained by an enhanced demand for NAD at low pH in order to remove the increased levels of reducing equivalents. However, biosynthesis of 3-methyl-1-butanol also involves oxidoreductase steps [18]. The increased expression of genes involved in vitamin biosynthesis and other coenzyme biosynthesis (nicotinamides) at low pH strongly suggests that these metabolic functions contribute to the BCAA-metabolism, stimulating production of 3-methyl-1-butanol. Elevated gene expression of genes involved in nicotinamide pathways may also be linked to the

redox-step involved in the conversion of the branched chain aldehyde, e.g. 3-methyl-1-butanal to the fusel alcohol, 3-methyl-1-butanol. Since it is known already that the uptake of BCAA's, the transaminase or the decarboxylase steps are not the rate-limiting steps in fusel alcohol formation, the reduction step (essential for the formation of 3-methyl-1-butanol from L-leucine) thus is an important target for construction of improved strains.

The other GO groups identified are involved in the glutamate metabolism and tricarboxylic acid (TCA) cycle. 2-Oxoglutarate, the amino-acceptor, is required for synthesis of glutamate. Glutamate is the amino-donor in the BCAA biosynthesis, and both are thus essential for fusel alcohol synthesis. Citrate is converted to 2-oxo-glutarate by the TCA cycle enzymes, involving the NAD-dependent isocitrate dehydrogenase step. Our results show that the genes *CIT1, CIT2* have an elevated expression at pH 3.0, possibly reflecting that availability of 2-oxoglutarate precursor plays a crucial role in the fusel alcohol production (Fig. 6). The activity of the *KGD1* and *KGD2* genes was reduced at pH 3.0. Therefore, *BNA6, BNA2, BNA4, BNA3, YOR356W, RPE1, MDH3, CIT1, CIT2, KGD1* and *KGD2* are additional target genes for future studies.

In conclusion, the high throughput microtiter screening was successfully applied in combination with cDNA microarrays to gain insights into molecular processes during specific process conditions. Investigation of environment-gene interactions for identification of key enzyme steps, or regulatory pathways, is paramount for functional genomics, revealing targets for directed mutagenesis. The selected genes are promising targets for the development of improved production strains. This specific example illustrates that the MTP screening assays (a) allowed a qualitative profiling of the effects of changes in extracellular conditions on fusel alcohol formation, (b) allowed rapid identification of optimal process condition for fusel alcohol formation in yeast, (c) provided a firm base for subsequent cDNA microarray gene expression analysis. Both, the high-throughput MTP approach and the genomics can be used to analyse any fermentation process, even with other micro-organisms.

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Chapter Six

Genome-wide expression analyses on flavour formation in retrospect

Genome-wide expression analyses on flavour formation in retrospect

The food industry is a market that has developed considerably over the last few decades. Fermented foods produced throughout the world, comprise about 25% of our food intake. An example of a popular fermented food condiment is soy sauce. The production of soy sauce involves a two stage process. The first stage is known as koji (soybean mash) and is similar to the process involved in brewing. This stage is followed by a fermentation process, in which amino acids, derived from the koji, are converted in aroma compounds by micro-organisms [1]. Soy sauce fermentation encompasses a complex interaction between micro-organisms and their environment. The influence of the environment can immediately be connected with changes in the metabolism of the micro-organisms. In the soy sauce fermentation, the yeast *Zygosaccharomyces rouxii* has been identified as the major contributor to the final taste of the sauce. It is responsible for the generation of characteristic flavour compounds; ethanol, fusel alcohols and 4-hydroxyfuranones [2, 3]. Yet, the final taste quality is difficult to control and an in-depth knowledge of yeast fermentation is prerequisite. Since the fusel alcohol 3-methyl-1-butanol has been thought to have major influence on the specific taste of fermented foods, the knowledge of molecular mechanism involved in 3-methyl-1-butanol production would be of great interest in controlling and ultimately accelerating the flavour forming process.

We have set up original experimental models to assess the gene response specific to 3-methyl-1-butanol producing conditions (growing on the nitrogen source L-leucine). We used a DNA-microarray-based approach to identify genes that are up or down regulated under such conditions and show that genes of different pathways, other than specific BCAA pathways were strongly induced or diminished. Furthermore, gene promoter analyses were carried out to examine the observed expression behaviour.

The use of microarrays has become widespread since their first appearance 8 years ago. Analyses as described in chapter 2 were just at the beginnings of microarray data analyses. Chapter 2 describes a comparative experiment, where replicate arrays were used to elucidate transcription profiles of *Z. rouxii*. Spot values of the array, hybridized with labelled *Z. rouxii* cDNA, plus a simple expression ratio analysis were executed. Our main concern was to choose an experimental microarray and image processing design. Normalisation was

performed, based on northern blot analysis, by comparing gene expressions with 'household genes'. Statistics was solely applied to control the hybridization quality of the spots. Spot quality was determined by 3 criteria; 1) spot intensity $(M_s-M_b)\cdot(\sigma_b)^{-1} > 0.5$, where M_s stands for the mean of the signal, M_b stands for the mean of the background, and σ_b stands for the standard deviation of the background, 2) $(\sigma_s)\cdot(M_s)^{-1} < 0.2$, where σ_s stands for the standard deviation of the signal and 3) $M_s > M_b$. This was validated for the desired purpose, the investigation of filter crosshybridization. We were able to determine 115 *Z. rouxii* genes by this approach. However, no genes related to flavour formation were revealed. The power of crosshybridization was affirmed 2 years later, where crosshybridization was used to determine yeast species. Crosshybridization of labelled genomic DNA on Yeast Chips provided more rapid and convenient information on the genome of a sample yeast strain than general DNA-DNA hybridization methods commonly used [4].

In the following chapters we investigated the correlation between changes in gene expression and the 3-methyl-1-butanol appearance. More refined statistics were desired for the justification of conclusions on gene expression changes. The central question we encountered after assaying cDNA microarrays was how to analyse the datasets resulting in the identification of differentially expressed genes.

In chapter 3, gene disruption experiments were carried out to examine the role of *BAT1* and *BAT2* in the flavour formation. Gene expression profiles of wild type yeast were compared with 3 deletion mutant strains, under 3-methyl-1-butanol producing conditions. In addition, strain cultivations were performed on either glucose or ethanol. It became clear that the analytical aspects of microarray datasets were complicated. Specific tools were needed to handle the huge datasets in a correct way. Experiments were performed in triplicate in order to apply the statistical analysis tool, one-way ANOVA, for identifying significant gene expression between the pairs of conditions. It appeared that SPSS, the standard statistic program, had difficulties in handling such mass of data. However, during the project, rapid progresses in the field of bioinformatics led to the development and availability of specific microarray analyses tools. The SAM (Significance Analysis of Microarray) Test, based on a method developed by Tusher, Tibshirani, and Chu [5] and described by Storey [6], became freely available. The SAM test used repeated permutations of the data to determine if the expression changes of any genes were significantly related to the response. The program created a profile of observed versus expected values. Values, lying

outside the defined region of this profile, were considered as genes significantly related to the investigated condition. Moreover the program was able to handle multi-class responses, such as those generated in our experiments. By using this program from that moment on, the presence of false positives was minimized. We defined a SAM-calculated q-value, with q < 5% as threshold for identification of differentially expressed genes. The q-value represented the false positive chance of the hybridization spot. Figure 1 shows the SAM significance plot for the yeast strains cultivated on ethanol, from data of chapter 3. The selected genes had an altered expression pattern due to the deletion of the genes *BAT1, BAT2* or both genes.

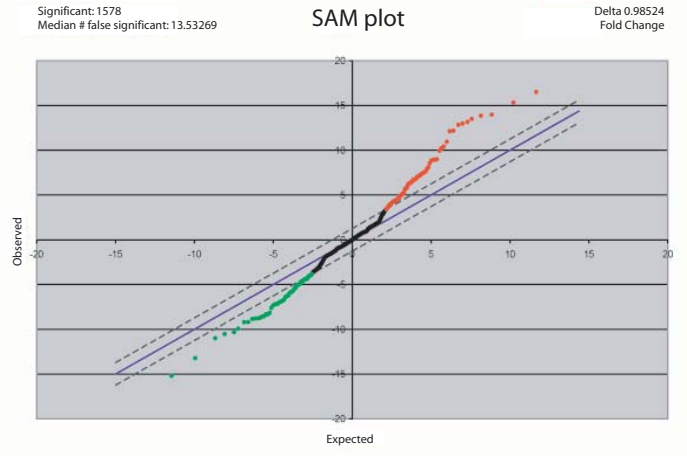

Figure 1. SAM-plot representing the score of each gene in the real distribution compared to the random distributions from the dataset wild type and mutant yeast strains grown on ethanol (chapter 3). The differentially expressed genes are the ones moving away from the 45° slope line.

The first available web based programs 'Cluster' and 'Treeview' [7], to extract and visualize gene clusters were also used in chapter 3. With these programs cluster analysis, hierarchical clustering (average linkage clustering) was applied. Hierarchical average linkage clustering exposed 2 different gene clusters for *BAT1* and *BAT2*. The gene *BAT1* encoding mitochondrial branched chain amino acid permease was induced in response to fermentation in presence of glucose whereas *BAT2* encoding cytosolic branched chain amino acid permease was induced in response to growth on ethanol. In addition hierarchical cluster analysis revealed that in the *BAT2*-cluster the STRE-element appeared in a great extent.

As we used MIPS datasheets in combination with our array data in chapter 2, a convenient web based program, SGD GO TermFinder became available for microarray data analysis [8, 9]. This program has the ability to determine rapidly significant GO annotations for a list of genes, generated by microarrays and facilitated the elucidation of cellular pathways, which the significant altered genes had in common. The application of self-organizing maps (SOM), a type of mathematical cluster analysis that was particularly well suited for recognizing and classifying features in complex, multidimensional data, provided pictures for data visualization (Fig. 2A). In contrast to data analysis visualizations like self-organizing maps, SGD GO TermFinder provided visualization of gene expression changes in an easy comprehendible flow diagram linked to metabolic functions (Fig. 2B). As a result we were able to visualize the involvement of genes of pathways, other than the BCAA pathways, in flavour production from the cDNA microarray data as described in chapter 4.

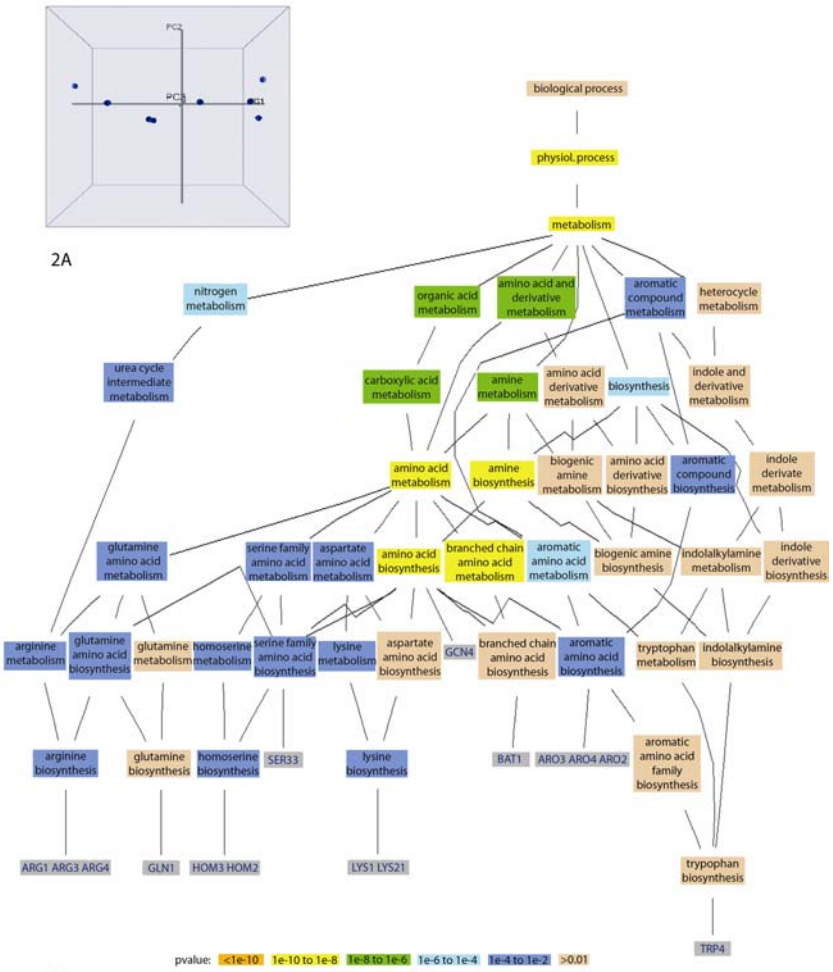

Figure 2A-B. Visualizing output from microarray data, investigating L-leucine metabolism, cells cultivated on either ammonia or L-leucine (chapter 4). A) Principle of Self-organizing maps (SOMs) of gene expression data of the 3-methyl-1-butanol cluster. Nodes of SOM are 3-dimensional represented by dots and the initial geometry of nodes is shown in a rectangular grid. B) Principle of GO TermFinder. GOgraph represents the significant GO nodes of the 3-methyl-1-butanol cluster, which explicitly showed involved genes: *ARG1, ARG3, ARG4, GLN1, HOM3, HOM2, SER33, LYS 1, LYS 21, BAT1, ARO3, ARO4, ARO2* and *TRP4*. The color of the nodes is an indication of the Bonferroni corrected *P*-value (orange <= 1e-10; yellow 1e-10 to 1e-8; green 1e-8 to 1e-6; cyan 1e-6 to 1e-4; blue 1e-4 to 1e-2; tan > 0.01) (see colour version at page 139).

In chapter 4 we started to use an additional microarray analysis web-tool SNOMAD (Standadization and NOrmalization of MicroArray Data) for the normalization of the array data. The SNOMAD-tool calculated element signal intensities to a mean intensity locally across the surface of the DNA microarray [10]. Hereby we were able to reduce spatially systematic artifacts in the data, introduced during microarray-steps such as robotic printing, hybridization, washing, or imaging of the arrays (Fig. 3). The data was SNOMAD normalised prior to the SAM-test. The group of genes with significantly changed expression was subsequently analysed with Regulatory Sequence Analysis Tools (RSAT) [11]. This resulted in the identification of two gene-groups, containing an upstream activation sequence (UAS) for Gcn4p or Gln3p. Since the compared gene expression data were obtained via growth on different nitrogen sources and Gcn4p and Gln3p are well-known transcription factors involved in nitrogen regulation we were convinced of the necessity of using SNOMAD normalisation prior to data-mining.

Originally designed to study the 3-methyl-1-butanol production in context of soy sauce fermentation, the experimental models established in this thesis showed a direct correlation between the 3-methyl-1-butanol concentration in the medium and affected gene expression levels of specific genes. Identified genes hold a great promise in leading us closer to a complete picture of flavour formation by yeast (Fig. 4). Additional hand-on experiments are needed to learn about the exact gene functions. As through this project, the field of genomics is still rapidly evolving. Not only genome-wide gene expression analysis, also genome-wide protein analysis, enzyme activity analysis, protein localisation studies and genome wide metabolite profiling [12, 13, 14, 15, 16, 17] are advancing. In chapter 5 we studied 3-methyl-1-butanol formation with high throughput automated metabolite screenings assays in combination with cDNA microarrays. Although these data are currently incomplete and error-prone, they provided a glimpse of a new view on flavour formation, derived from the large datasets.

In closing, to control the final taste quality of a fermented food, like soy sauce, yeast genomics revealed genes involved in pathways, apparently unrelated to 3-methyl-1-butanol production, which are of great interest for future research on controlling and ultimately accelerating the flavour forming process in fermented foods.

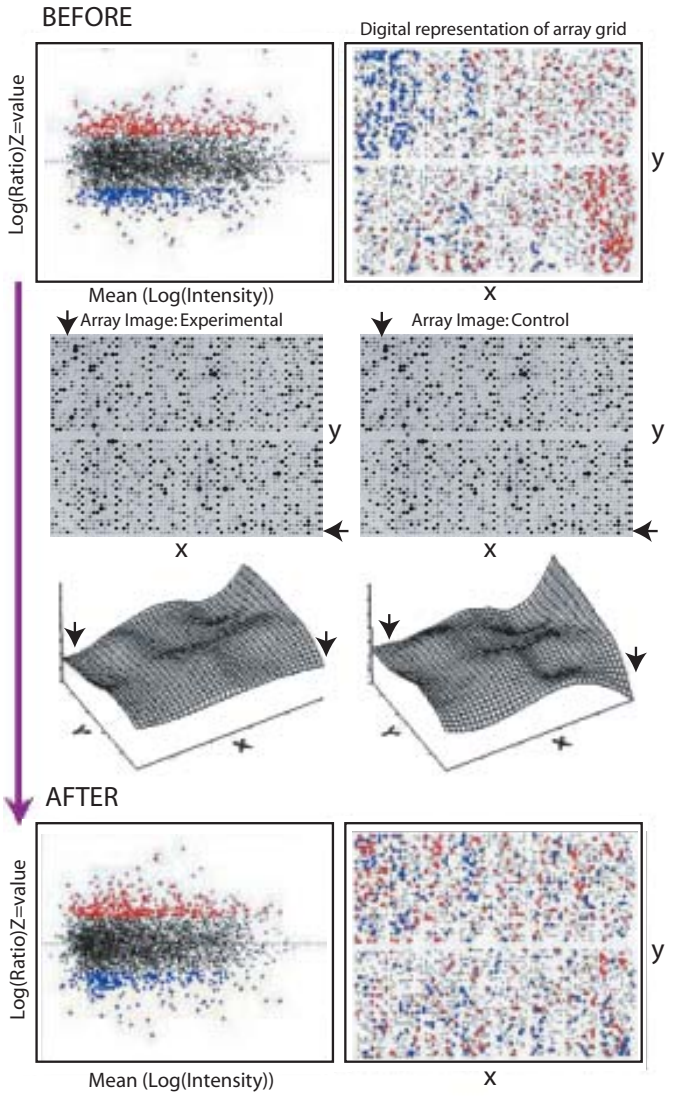

Figure 3. SNOMAD correction on artifacts like non-uniform background intensities on the surface of the microarray. Picture is taken from http://pevsnerlab.kennedykrieger.org/

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Summary

Summary

It has been established that L-leucine consumption by yeast leads to the appearance of the organoleptic substance 3-methyl-1-butanol. This substance has a major role on the final flavour of fermented foods. Information about the underlying molecular mechanisms on 3-methyl-1-formation is scarce. The formation of metabolites has its roots in the genomic information. Therefore, identification of the enzymes and genes involved is a major prerequisite to enable optimisation and control of a desired blend of flavours.

In the 3-methyl-1-butanol pathway, two ORFs have been identified, *BAT1* and *BAT2*, encoding enzymes responsible for the first step in the L-leucine breakdown. There was no repression of 3-methyl-1-butanol production when one or both of the enzymes were deleted. The amount of 3-methyl-1-butanol was also not drastically changed when the uptake of L-leucine was increased by several amino acid permease gene overexpressions. Studies involving the influence of pyruvate decarboxylase genes led to the conclusions that genes coding for pyruvate decarboxylases, *PDC1, PDC2, PDC3* and *PDC6,* were expendable for 3-methyl-1-butanol formation.

The development of cDNA-microarrays and the availability of arrays containing immobilized probes of all yeast open reading frames, offered us the possibility to look at transcription activity genome-wide. We started to investigate the genome-wide expression in the 'soy yeast' *Zygosaccharomycs rouxii*. We determined that GeneFilters®, containing *S. cerevisiae* ORFs, were useful in the elucidation of 155 *Z. rouxii* genes, chapter 2. We established that the yeast GeneFilters® were able to discriminate the expression of genes with a DNA sequence homology up to 83%. Due to this discriminating capacity of the GeneFilters®, it was not surprising that only a relative small group of genes (155 out of ~5000 Zr ORFs) was detected by crosshybridization.

Additional knowledge of *Z. rouxii* transcripts was obtained by comparing salinity stressed *Z. rouxii* cells with non-stressed cells. To set a comprehensible and reproducible starting point, the level of significance of changes was set arbitrarily with a factor 3. Using this criterion, 86 *Z. rouxii* genes were found in responds to osmostress. 67% of these genes showed similar gene expression ratios compared with *S. cerevisiae*. We observed that the response of genes like *HMO1* (required for normal growth and for plasmid maintenance), and other genes involved in protein synthesis, metabolism and cell growth, were

substantially upregulated in *Z. rouxii* in the presence of NaCl. This indicated halophilicity of the yeast *Z. rouxii,* which was also observed in classical growth experiments. Of course, one can imagine situations in which a three-fold change in mRNA level may have little physiological consequence, and others where a low change may be critical. However, crosshybridization provided us novel clues for genes present in *Z. rouxii* and demonstrated to be a rapid detection method to gain insight in gene expression profiles for this yeast genus. Yet, the number of elucidated genes was too low for continuation of crosshybridization experiments in the context of 3-methyl-1-butanol formation.

Chapter 3 describes genome-wide expression analyses in *S. cerevisiae*. We demonstrated that Bat2p is essential for fusel alcohol production in cells grown on ethanol. When *BAT2* was deleted, no 3-methyl-1-butanol was produced by *bat2Δ* - and *bat1Δ bat2Δ*-deletion strains. *BAT1* and *BAT2* showed different expression patterns in *S. cerevisiae* during either growth on glucose or on ethanol. Transcription activities of *YDL080c,* encoding a pyruvate decarboxylase-like gene and *ADH1,* encoding an alcohol dehydrogenase, were declined under the conditions no 3-methyl-1-butanol was formed. Deletion of *BAT1* or *BAT2* caused in cells cultivated on glucose, an increase in transcription activity of genes encoding transaminases: *ARO9, BNA3, GFA1, SNO1,* and *SNO3*. Three genes, *HIS5, SER1* and *SNO3*, showed an enhanced expression by *BAT1* deletion in cells cultivated on ethanol. Strikingly, no significant enhanced gene expression of transaminases was observed when *BAT2* was deleted in ethanol cultivating cells. In addition hierarchical clustering of array data revealed that *BAT2* was co-expressed with several genes containing one of more *STRE*-elements. Since *BAT2* itself possesses 2 *STRE*-regulatory elements, one could speculate that these *STRE*-regulatory elements may be of importance for the regulation of *BAT2*.

With these results we clearly determined that, although *S. cerevisiae* possesses two highly homologues BCAAT isozymes Bat1p and Bat2p, *BAT1* and *BAT2* have different expression patterns in cells during the conditions tested. Also the *bat1Δ* and *bat2Δ*-deletion strains behaved differently compared to each other. It is tempting to speculate, based upon gene expression patterns that other enzymes are able compensate the loss of the branched chain amino acid transaminases under certain circumstances.

After the transaminase step, a decarboxylation and a reduction step complete the leucine degradation (Ehrlich) route. In Chapter 4, genomic studies to clarify

the production of 4 volatile flavour substances produced by *S. cerevisiae,* 4-methyl-2-oxopentanoate, 3-methyl-1-butanol, 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid, are described. The appearance of these compounds could not simply be explained by the Ehrlich route. Microarrays revealed that beside genes involved in branched chain amino acid biosynthesis, genes classified in amino acid biosynthesis, amine biosynthesis, caboxylic acid- and organic acid metabolism, were altered in expression level during flavour producing conditions. Interestingly, non-branched chain amino acids: aromatic amino acids, serine, arginine, glutamine, homoserine and lycine, superficially unrelated with branched chain amino acids, were also effected when L-leucine was used as nitrogen source. To reduce biological noise, caused by dynamic growth conditions, which cells face during batch fermentation, we also cultivated cells in chemostats under continuous fermentation conditions. Hereby we compared gene profiles of shakeflask cultures with profiles of cells cultivated in the chemostats. We identified a group of 117 genes, altered in expression irrespectively of batch or continuous growth conditions. Among this group, a subgroup of genes was significantly present, which contained regulatory elements for *GCN4* and *GLN3*. *GCN4* and *GLN3* encoding transcription factors involved in nitrogen metabolism. Additionally, our results showed that *BAP2* and *BAP3*, encoding permeases necessary for the uptake of L-leucine, *PDX1* and *YDL080c,* encoding decarboxylases for 4-methyl-2-oxopentanoate and *ADH3, ADH5, YMR318c* and YCR105w, encoding alcohol dehydrogenases appeared to be upregulated in *S. cerevisiae* cells. It is also worth noticing that two genes homologous to leucine degradation genes in mammals and bacteria *YGL059w* and *ACH1* were part of the 117 genes. Concluding from the gene analysis we presumed that this specific gene group is involved in flavour formation, irrespective of growth conditions. Reverting to the soy yeast *Z. rouxii,* it has been shown that the presence of threonine in the medium interfered with the formation of 3-methyl-1-butanol. This finding compromises the observation that genes of different metabolic routes in the cell are altered during flavour formation and that products like 3-methyl-2-oxobutanoic acid and 3-methylbutyric acid emerged. The combination of metabolite formation and the expression data from cDNA microarrays provided strong indications of the existence of alternative pathways. Furthermore, comparing different cultivation methods and searching for overlapping gene expression profiles proved to be an useful tool for data extraction.

Another innovative method to extract data is described in chapter 5. The goal of this study was to elucidate target genes for strain improvement, by combining

results obtained by spectra photometric, HPLC and GC assays with genome-wide expression datasets. Metabolic-profiling technologies were applied to study the correlation of 3-methyl-1-butanol to the process conditions pH, and salt. The variables pH and NaCl-concentration were selected, given the fact that many food fermentations include specific pH and salt-conditions. To invest a pH range of pH 3.0 – pH 7.0 integrated with an NaCl-concentration range of 0 – 10% w/v simultaneously in a short time, we performed small scale fermentations in microtiter plates (MTP). This approach allowed us to screen 96 simultaneous micro cultivations. Prior to our research, the use of MTPs was validated for 3-methyl-1-butanol formation in *Z. rouxii*. We confirmed that automated MTP fermentations performed in sealed round deep well MTPs gave a rapid and reliable identification of the effect of pH or/and NaCl on 3-methyl-1-butanol formation in *S. cerevisiae*. We established that the presence of NaCl had an inhibitory effect on 3-methyl-1-butanol formation in *S. cerevisiae* and that an acidic environment (< pH 5.0) amplified the inhibitory effect of NaCl on the fusel alcohol formation. The highest fusel alcohol formation was measured at pH 3.0 without NaCl. Under this condition cells were cultivated in 500 ml erlemeyers and harvested for cDNA microarray analyses. The gene expression profile was compared with cDNA microarray data of cells, cultivated under similar conditions at the standard pH 5.0.

The cDNA microarrays showed 17 highly active genes, encoding steps in BCAA metabolism. A subset of 9 genes, *BAP2, BAP3, LAT1, PDX1, THI3, ALD4, ILV3, ILV5* and *LEU4,* displayed a change in gene activity at pH 3.0. In total 747 genes displayed significantly altered transcription activity at pH 3.0. Further investigation on this dataset by the web-tool SGD Gene Ontology (GO) Term Finder, revealed that a significant part of the genes in this group had a function in the metabolism of nicotinamides, vitamins, fatty acids, glutamate or carboxylic acids. From this group, 11 genes, *BNA6, BNA2, BNA4, BNA3, YOR356W, RPE1, MDH3, CIT1, CIT2, KGD1* and *KGD2,* were appointed as promising targets for the development of improved production strains, based on their altered gene expression at pH 3.0 and literature research. Further experiments are crucial for establishing the weight of these identified genes.

Nederlandse samenvatting

Nederlandse samenvatting.

Micro-organismen spelen een cruciale rol in de smaakvorming van gefermenteerd voedsel. De bakkersgist, *Saccharomyces cerevisiae,* is het meest bekende micro-organisme, dat al ver voor de jaartelling gebruikt werd voor het bereiden van brood, wijn en bier. Een andere gist, *Zygosaccharomyces rouxii,* is verantwoordelijk voor de 'fruitige' smaak in sojasaus (ketjap). Beide gisten zetten vertakte aminozuren om in vertakte aldehydes, zuren en alcoholen, die uiteindelijk zorgen voor de specifieke smaak. De gist zorgt dus bij het bakken van brood niet alleen voor het rijzen van het deeg, maar draagt ook in grote mate bij aan de smaak ervan. Het verkorten van de rijstijd zoals bij fabrieksbrood, heeft invloed op de smaak (kwaliteit) van het brood. Door de verkorte rijstijd mist het een aantal belangrijke geur- en smaakcomponenten. Het bereiden van traditioneel sojasaus kent een nog langer vergistingsproces van ongeveer 6 maanden. Gedurende deze tijd maken gisten (*Z. rouxii)* smaakstoffen vanuit gehydrolyseerde sojabonen. Om de procestijd te verkorten worden in sojasausen vaak petrochemische smaakstoffen toegevoegd. Een groot nadeel van het "chemisch" produceren van sojasaus is de eventuele aanwezigheid van carcinogene verbindingen, zoals chloropropanols. Dit is de rede dat een aantal ketjap-merken in 2001 uit de handel zijn genomen. Bovendien is het verplicht volgens de voedselwetgeving, toegevoegde petrochemische smaakstoffen in levensmiddelen op het etiket te vermelden als kunstmatige smaakstoffen. Door de vraag naar gezond, veilig en biologisch voedsel is er (hernieuwde) belangstelling voor smaakstof vorming door gisten.

Diverse onderzoeken hebben aangetoond dat de smaak van gefermenteerd voedsel te beïnvloeden is door middel van veranderde procescondities, zoals temperatuur, zoutgehalte en pH. Het onderzoek beschreven in dit proefschrift, richt zich voornamelijk op de metabole routes in de gistcel zelf. Het is bekend dat de typisch gefermenteerde smaak veroorzaakt wordt door foesel alcoholen, ook wel foesel oliën, hogere alcoholen of vertakte alcoholen genoemd. Het onderzoek werd gestart om genen te identificeren die een rol spelen in de omzetting van het vertakte aminozuur L-leucine naar de vertakte alcohol 3-methyl-1-butanol, met uiteindelijk als doel een giststam te maken die een verhoogde hoeveelheid foesel alcoholen vormt tijdens de fermentatie. Aan het begin van dit onderzoek waren slechts twee genen bekend, *BAT1* en *BAT2,* als zijnde betrokken bij deze metabole route. Deze genen coderen voor enzymen die verantwoordelijk zijn voor de eerste stap in de omzetting van L-leucine naar 3-methyl-1-butanol. De ontwikkelingen op technologisch vlak hebben ons de

mogelijkheid geven inzicht te krijgen in het expressie profiel van alle ~6000 gist genen tegelijkertijd. Dit is mogelijk met behulp van cDNA microarrays. Hiermee wordt er een zogenaamd 'genoomwijd' beeld verkregen van actieve en inactieve genen en van de mate van hun activiteit. Dit proefschrift beschrijft de genoomwijde studie van de smaakstofvorming door gisten, een nieuwe stap naar het begrijpen van 3-methyl-1-butanol vorming.

In hoofdstuk 2 worden crosshybrydisatie experimenten beschreven voor het in kaart brengen van *Z. rouxii* genen. Met behulp van cDNA GeneFilters® is de expressie van verscheidene *Z. rouxii* genen gedetecteerd. Om de expressie van de genen te bepalen werden er radioactieve cDNA probes gesynthetiseerd van geïsoleerd *Z. rouxii* mRNA. Deze probes werden gehybridiseerd met de GeneFilters®. Tevens werd de gen expressie in de gisten bestudeerd, die bloot waren gesteld aan zout. Het effect van NaCl werd bestudeerd, omdat veel voedsel fermentaties, met name sojasaus, plaatsvinden in een zoute omgeving. Om vast te stellen of gen-expressie beïnvloed werd door de aanwezigheid van NaCl, werd een expressie verschil gehanteerd van een factor 3 of groter als grenswaarde voor een significante verandering in gen-activiteit. In totaal werden er op deze wijze 155 *Z. rouxii* gene geïdentificeerd, waarvan 86 genen in expressie veranderden als er zout in het medium aanwezig was. 67% van de genen vertoonden eenzelfde expressie gedrag als de genen van de bakkersgist *S. cerevisiae,* ten gevolge van de zoutstress. 33% van de geïdentificeerde *Z. rouxii* genen gedroeg zich anders ten opzichte van *S. cerevisiae*.

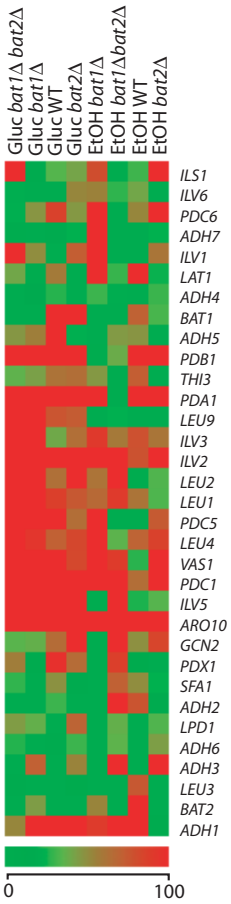
Geen van de 155 geïdentificeerde genen konden gerelateerd worden aan het vertakte aminozuur-, algemene aminozuur- of stikstofmetabolisme. Ook genen coderend voor enzymen van andere bekende routes, zoals de glycolyse of citroenzuurcyclus konden niet worden gedetecteerd op de GeneFilters®. Aan de hand van hybridisaties met *S. cerevisiae* probes constateerden we dat de filters een discriminerend vermogen hadden voor genen die tot 80% homoloog zijn. Dit discriminerend vermogen verklaart de detectie van relatief weinig spots (155 van de ~5000 *Zr* genen) op de filters met de *Z. rouxii* probes. Uit deze experimenten concludeerden we dat crosshybridisation een manier is om bepaalde genen van *Z. rouxii* op te sporen, maar dat de gisten teveel van elkaar verschillen om met behulp van GeneFilters® een genoomwijd beeld van *Z. rouxii* te creëren. In de volgende hoofdstukken worden de 'genomics' op *S. cerevisiae* beschreven. Het gen expressieprofiel van *S. cerevisiae* kan naast het feit dat deze gist gebruikt wordt in vele voedselfermentaties, mogelijkerwijs ook als voorbeeld dienen voor smaakstof vormende routes in *Z. rouxii*.

In hoofdstuk 3 wordt de afzonderlijke invloed van de genen *BAT1* en *BAT2* in *S. cerevisiae* met betrekking tot de foesel alcohol 3-methl-1-butanol beschreven. Dit hebben we onderzocht met behulp van gen-deletie experimenten. Beide genen coderen voor enzymen (Bat1p en Bat2p) die de conversie van L-leucine naar 4-methyl-2-oxopentanoate catalyseren. Er is tevens gekeken naar gen-expressie bij groei op glucose en op ethanol. In de eerste fase van de fermentatie is er voor de gist glucose in overvloed en consumeert hij de aanwezige glucose, die wordt omgezet in ethanol. In de tweede fase, waar er weinig tot geen glucose meer aanwezig is, groeit de gist verder op ethanol. Met behulp van cDNA microarrays werd duidelijk dat de genen *BAT1* en *BAT2* ieder een eigen expressie patroon vertoonden in *S. cerevisiae*. We hebben laten zien dat *BAT2* sterk in de gist tot expressie komt, als deze ethanol als koolstofbron gebruikt. Op het moment dat *BAT2* niet meer in de cel aanwezig is, zoals in de *bat2Δ* - and *bat1Δ bat2Δ*-deletie stammen, werd er geen 3-methyl-1-butanol meer geproduceerd. Transcriptie activiteiten van de genen *YDL080c* (coderend voor een pyruvate decarboxylase) en *ADH1* (coderend voor een alcohol dehydrogenase) waren onder deze condities ook significant afgenomen. Hieruit concludeerden we dat het gen *BAT2* noodzakelijk is voor de smaakstof vorming (3-methyl-1-butanol) gedurende op ethanol. De afwezigheid van de *BAT*-genen had geen invloed op de smaakstof vorming gedurende gist fermentatie op glucose. Op de cDNA microarrays was te zien dat genen coderend voor verschillende transaminases *ARO9, BNA3, GFA1, SNO1* en *SNO3*, verhoogde gen activiteit vertoonden in de *bat1Δ-, bat2Δ* - en *bat1Δ bat2Δ*-deletie stammen. In de *bat1Δ*-deletie stam groeiend op ethanol, werd een verhoogde activiteit waargenomen voor de transaminases *HIS5, SER1* en *SNO3*. In de *bat2Δ* - and *bat1Δ bat2Δ*-deletie stammen op ethanol, werden geen significant verhoogde gen expressies waargenomen voor de transaminases. Dit duidt erop dat er geen back-up transaminase aanwezig is op het moment van een *bat2*-deletie stam en groei op ethanol. Naast deze observaties werden er met behulp van een hierarchische clusteringsprogramma Cluster 2 groepen genen geïdentificeerd uit de array data, die eenzelfde expressie patroon vertoonden als *BAT1* of *BAT2*. Analyse van de promotor regionen van de gen-groepen wees uit dat de meerderheid van de genen die tot co-expressie kwamen met *BAT2,* 1 of meerdere stress responsieve elementen (STREs) bevatten. Ook *BAT2* zelf bevat 2 STREs in de promotor regio. Verder onderzoek aan deze elementen zal moeten uitwijzen hoe belangrijk de STREs zijn voor de regulatie van *BAT2* en de uiteindelijke smaakstof vorming.

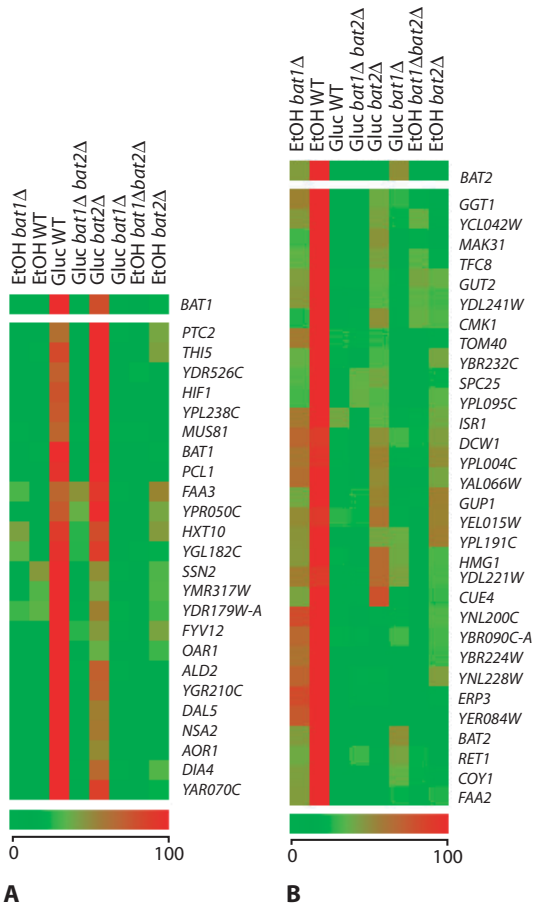
Om een genoomwijd gen-expressie beeld te krijgen van de smaakstof vorming uit vertakte aminozuren in de gistcel, is naast het genoomwijde gen-expressie profiel van de *bat*-deletie stammen, het genoomwijde gen-expressie profiel van gisten gegroeid op L-leucine en dat van ammonium in kaart gebracht. De resultaten van de genoomwijde analyses zijn beschreven in hoofdstuk 4. De gisten, gegroeid op de simpele stikstofbron ammonium, produceerden geen waarneembare smaakstoffen. Voor de analyses werd *S. cerevisiae* gekweekt in erlemeyer schudkolven van 500 ml (batch-groei). De vergelijking van de gen-expressie profielen van gist gegroeid op L-leucine of ammonium leverde 930 genen op, die in activiteit veranderden op het moment dat er smaakstoffen gevormd werden. Eenzelfde proef, uitgevoerd in een chemostaat (ook wel continuous stirred tank reactor, CSTR, genoemd), leverde 752 genen op met significant veranderde activiteiten. Een vergelijking tussen de gen-ratio's van de erlemeyers en de CSTR resulteerde in 117 overlappende ratio-waarden. We hebben vastgesteld dat in deze groep van 117 genen de regulatoire elementen voor Gcn4p en Gln3p significant vaker voorkwamen dan andere regulatoire elementen. Gcn4p en Gln3p zijn bekende *S. cerevisiae* transcriptie factoren, betrokken bij het stikstof metabolisme. Promoter analyses van de overige genen niet behorend tot de '117-groep', leverden geen opvallende regulatoire elementen op. Hierarchische clustering van de totale genoomwijde datasets resulteerde in 2 clusters: een cluster van de in erlemeyers gecultiveerde gist en een cluster van de CSTR gecultiveerde gisten. Hieruit blijkt dat de manier van cultivering een grotere invloed heeft op de expressie van genen dan de af of aanwezigheid van L-leucine en vorming van smaakstoffen. Hierarchische clustering van de 117 genen, leverde ook 2 clusters op: een cluster met de CSTR L-leucine, batch L-leucine en batch ammonium gecultiveerde gistcellen tegenover het andere cluster bestaande uit de gen-expressies van de gist gecultiveerd in de CSTR met ammonium. In de '117-groep' had de stikstofbron een doorslaggevende invloed op de clustering. Tijdens de groei op L-leucine werden er 4 verschillende smaakstoffen gedetecteerd met behulp van HLPC en GC: 4-methyl-2-oxopentanoaat, 3-methyl-1-butanol, 3-methyl-2-oxobutaanzuur en 3-methylbutyriel zuur. In de genoomwijde gen-expressie is te zien dat naast de genen die direct betrokken zijn bij het vertakte aminozuur metabolisme, ook de expressie van genen behorend tot andere functie groepen beïnvloed is tijdens smaakvorming. Belangrijke functie groepen die genen bevatten met veranderde activiteiten, waren de groepen van het niet-vertakte aminozuur metabolisme (aromatische aminozuren, serine, homoserine, arginine en lysine) en het carboxylzuur metabolisme. Het complete beeld van de smaakvorming is dus niet lineair, maar vertakt zich ook naar andere functie groepen.

Om een mogelijkheid te creëren om optimale proces condities te bepalen voor gen-expressie analyses, hebben we een high throughput (HTP) metaboliet screenings assay opgezet, waarna aan de hand van die resultaten cDNA microarray experimenten werden uitgevoerd. De high throughput (HTP) metaboliet screenings assay houdt een vrijwel volledig geautomatiseerd proces in, waar gisten gecultiveerd werden in 96-wells microtiter platen. Iedere 'well' stelde een individuele mini batch fermentatie voor. De microtiterplaten werden vervolgens automatisch geanalyseerd met spectrofotometrie, HPLC en GC. De combinatie van deze 2 high throughput screeningsmethodes, hebben we getoest met een screening van 2 variabele procescondities, pH en zout, en het invloed daarvan op de 3-methyl-1-butanol productie. Dit is beschreven in hoofdstuk 5. De invloed van de proces variabelen pH en NaCl-concentratie op de 1-methyl-3-butanol vorming werd onderzocht, daar veel voedselfermentaties een pH en een zoutstap bevatten. Uit de HTP-screening bleek dat de aanwezigheid van zout een remmende werking op de 1-methyl-3-butanol vorming had. Een lage pH bleek juist bevorderlijk te zijn voor deze productie. cDNA microarray hybridisaties lieten zien dat 9 genes, die specifiek te maken hebben met het vertakte aminozuur metabolisme (*BAP2, BAP3, LAT1, PDX1, THI3, ALD4, ILV3, ILV5* en *LEU4*) een verhoogde expressie vertoonden bij pH 3.0. Daarnaast werd met behulp van de web-tool SGD Gene Ontology (GO) Term Finder de functiegroepen van het nicotinamide-, vitamin-, fatty acid-, glutamaat- en carboxylzuur metabolisme, geïdentificeerd als zijnde meest significant veranderd tijdens de verhoogde smaakvorming bij deze pH. Binnen de groepen hebben we 11 genen geïdentificeerd (*BNA6, BNA2, BNA4, BNA3, YOR356w, RPE1, MDH3, CIT1, CIT2, KGD1* en *KGD2*) met verhoogde expressie. Deze selectieve gen-groep vormt een aantrekkelijk doelwit voor verdere studie naar de smaakstof productie. We kunnen dus concluderen dat de HTP-screening, gevolgd door cDNA microarray experimenten is een zeer succesvolle methode is om optimale groei-omstandigheden te bepalen voor gen-analyses. Het verkregen resultaat uit de arrays is als het ware een 'ruwe schets' van de smaafstof vorming en reikt handvaten aan voor de mogelijkheid foesel alcohol productie te sturen. De uitdaging is nu om te bepalen wat de directe waarde van gen-expressie ten opzichte van de 3-methyl-1-butanol productie is. Toekomstige experimenten zullen moeten uitwijzen wat de exacte effecten zijn van de geïdentificeerde genen op de uiteindelijke smaak van gefermenteerd voedsel.

Appendices



CHAPTER 3: Figure 4. Expression matrix of genes, involved in the Ehrlich pathway. Strongly expressed genes are visualized in red, no detectable gene-expression is visualized in green. Other gene-expression values are visualized in colours between red and green. The expression matrix is produced by Cluster and Treeview [18].

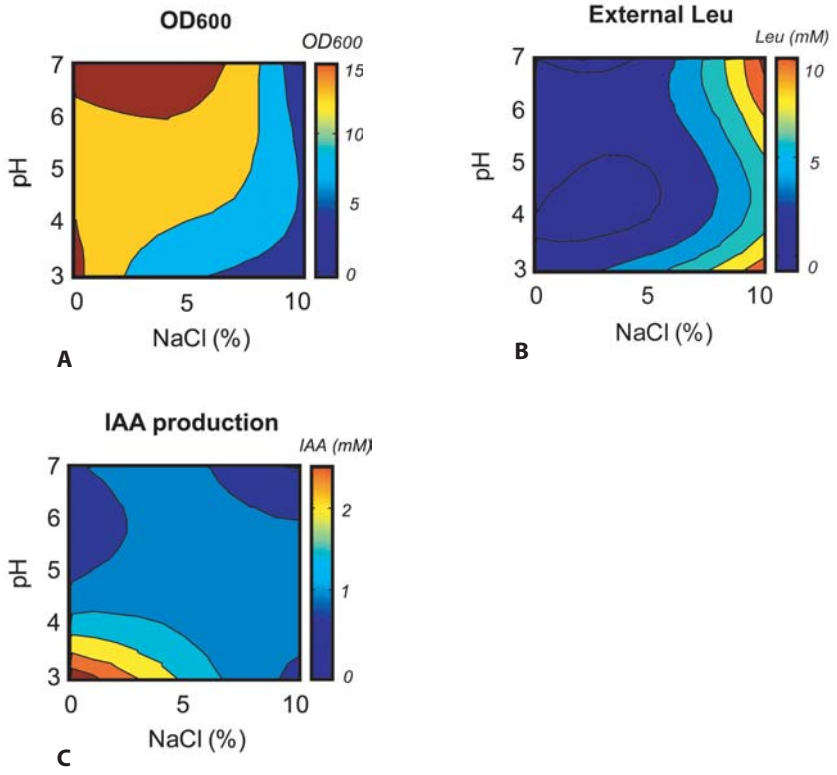


CHAPTER 3: Figure 6A-B. Average-linkage clustering. A) *BAT1* similarity matrix. All 24 genes in the *BAT1* cluster are strongly down regulated in the *bat1Δ*-mutant by growth on ethanol, compared to VWk43 grown on glucose. B) *BAT2* similarity matrix. *BAT2* cluster consists of 31 genes. These genes in the *BAT2* cluster are strongly down regulated when *BAT2* was deleted and when grown on glucose in comparison to VWk43 on EtOH. Both clusters consist for 50% of genes coding for unclassified proteins. In contrast to the *BAT1* cluster, the *BAT2* cluster is dominated by genes, which possess STRE elements.

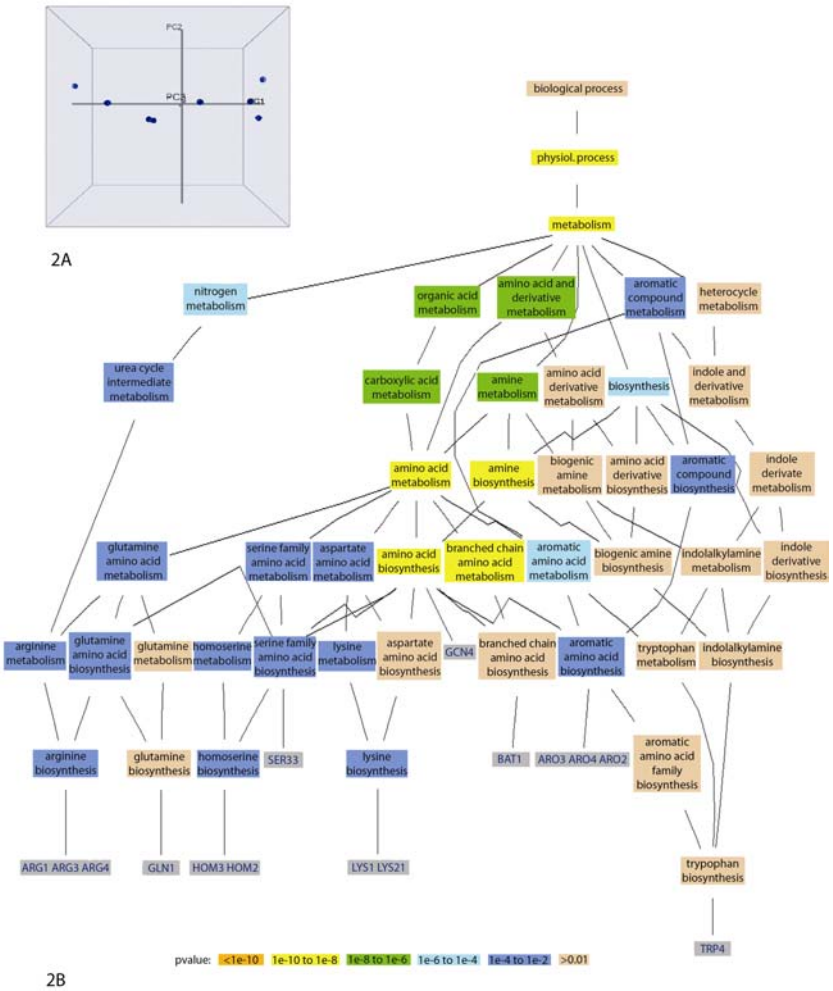
Useful weblinks:

ANOVA	http://www.jax.org/research/churchill/software/anova/
BioDiscovery	http://www.biodiscovery.com/
Brown Lab, Stanford University	http://cmgm.stanford.edu/pbrown/
Cluster/Treeview	http://rana.lbl.gov/EisenSoftware.htm
DeRisi Lab, University of California	http://www.microarrays.org/
EBI Microarray Informatics	http://www.ebi.ac.uk/microarray/
Eisen Lab	http://rana.lbl.gov/
GeneSpring	http://www.genespring.com/
Genomics Network for Young Scientists	http://www.geneyous.nl/
KEGG Kyoto Encyclopedia of Genes and Genomes	http://www.genome.jp/kegg/
MIPS – Comprehensive yeast genome database	http://mips.gsf.de/genre/proj/yeast/
Molecular Dynamics	http://www.mdyn.com

National centre for molecular biology information	http://www.ncbi.nlm.nih.gov/
Perl scripts	http://cellwall.stanford.edu/scripts
RCluster	http://genomics.biochem.uci.edu/genex/rcluster/
R packages	http://www.stat.berkeley.edu/users/terry/zarray/
Regulatory Sequence Analysis Tools	http://rsat.ulb.ac.be/rsat/
Resgen Yeast GeneFilters® Microarrays	http://www.resgen.com/products/YeastGF.php3
SAM	http://www-stat.stanford.edu/~tibs/SAM/
SGD - Saccharomyces Genome Database	http://www.yeastgenome.org/
SNOMAD gene expression data analysis tools	http://pevsnerlab.kennedykrieger.org/snomadinput.html
TIGR the Institute for Genomic research	http://www.tigr.org
YPD - Yeast Protein Database	http://www.proteome.com/
yMGV - yeast Microarray Global Viewer	http://www.transcriptome.ens.fr/ymgv/



CHAPTER 5: Figure 3A–C. Contour plots of *S. cerevisiae* grown in deep-well microtiter plates in medium with 6% glucose and 10 mM leucine at 30 ºC with final values of OD_{600} (A), L-leucine (B, mM),), 3-methyl-1-butanol (C, mM). Vertical bars indicate OD_{600} values or metabolite concentrations.



CHAPTER 6: Figure 2A-B. Visualizing output from microarray data, investigating L-leucine metabolism, cells cultivated on either ammonia or L-leucine (chapter 4). A) Principle of Self-organizing maps (SOMs) of gene expression data of the 3-methyl-1-butanol cluster. Nodes of SOM are 3-dimensional represented by dots and the initial geometry of nodes is shown in a rectangular grid. B) Principle of GO TermFinder. GOgraph represents the significant GO nodes of the 3-methyl-1-butanol cluster, which explicitly showed involved genes: *ARG1, ARG3, ARG4, GLN1, HOM3, HOM2, SER33, LYS 1, LYS 21, BAT1, ARO3, ARO4, ARO2* and *TRP4*. The color of the nodes is an indication of the Bonferroni corrected *P*-value (orange <= 1e-10; yellow 1e-10 to 1e-8; green 1e-8 to 1e-6; cyan 1e-6 to 1e-4; blue 1e-4 to 1e-2; tan > 0.01).

Over de schrijfster

Sung werd op 1 december 1973 geboren te Seoul, Zuid-Korea en groeide op in Kaatsheuvel, Noord Brabant. Ze volgde het Atheneum aan het Willem van Oranje College, te Waalwijk. Belangstelling voor de natuur, vogels en het leven zelf, deed haar besluiten biologie te gaan studeren. Zij studeerde biologie aan de Universiteit Utrecht van 1992-1997. In 1996 volgde ze met veel plezier een summer session van Liberal Arts and Sciences, aan de UCLA, University of California Los Angeles. Daarna begon ze met een 2-jarige postdoctorale opleiding Biotechnologie aan de Technische Universiteit Delft. De titel MSc Biotechnologie, met specialisatie industriële microbiologie, werd behaald in januari 2000. In maart 2000 startte ze als AiO met het Bedrijf Technologisch Samenwerkingsproject, genaamd "The improvement of the taste of fermented foods", aan de Universiteit van Utrecht. Dit promotie onderzoek, beschreven in dit proefschrift, werd uitgevoerd in samenwerking met Unilever Reseach Vlaardingen en de Rijksuniversiteit Groningen.

Nawoord

Nawoord

Bij deze wil ik iedereen bedanken die geholpen heeft bij het tot stand komen van dit proefschift. Allereerst wil ik mijn promotoren bedanken, de drie professoren Johannes, Arie en Theo. Jullie hebben het mogelijk gemaakt dit onderzoek te doen. Bedankt voor jullie vertrouwen in mij en alle ruimte die ik had om dit project vorm te geven. Johannes, het was echt super dat je altijd voldoende tijd wist te vinden om stukken door te spreken of na te kijken, zelfs met kerstmis (ha, ha, je zal me nog missen). De samenwerking heb ik als erg prettig ervaren en je nuchtere kijk op de wereld en humor heb ik zeer gewaardeerd (wat is het nut daar dan van?). Arie, ook jou wil ik graag bedanken voor het feit dat ik altijd bij je binnen kon lopen voor advies en voor het schenken van klare wijn in woelige tijden (wat een kwestie is van gewoon je boerenverstand gebruiken). Theo, met jouw aanwezigheid was het in ieder geval nooit saai. Je was een bron van inspiratie. Het was erg leuk dat je mee was op de gist-congressen in Praag en Madison. Ik heb ook zeer veel geleerd van de levendige discussies die we samen gevoerd hebben. Lubbert, als vierde prof was je betrokken bij mijn proefschrift. Hartelijk dank dat je, ondanks je drukke agenda altijd tijd had mijn mails te beantwoorden en stukken na te kijken. Deze samenwerking heb ik zeer gewaardeerd, samen met je poging om mij het 'komma'- gevoel bij te brengen. Je moet zelf maar beoordelen of het geholpen heeft. John, vanuit Unilever wist je altijd met opbouwende kritieken te komen. Hartelijk dank ook voor je begrip en de ruimte om tussen Unilever belangen en academische belangen in te balanceren.

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Victor (Victor, super dat je mijn paranimf wilt zijn). Bruno, arigatou gouzaimasu voor de foto's, Wally, de box doet het nog hoor. Van de studenten wil ik graag Arjan bedanken (helaas is je MSG werk niet in dit boekje terecht gekomen, maar hopelijk heb je het nog steeds bij Unilever naar je zin), Rick (wicked Rick), Chiel (sticky Foo), Megumi ('lekker kontje') en Lan. Zonder jullie aanwezigheid was er niet zoveel leven in de brouwerij geweest (en niet zoveel sushi en ander lekker eten...). Van de ondersteundende dienst, Antje, Ton en Cor, heel erg bedankt voor jullie hulp en interesse. Ayda, voor het schoonmaken en de belangstelling hoe het met mij ging. Marc, Mireille, Elsa, Anje, José, Ramon, Frits, Ilse, Rinse en Irina, bedankt voor de gezelligheid ook buiten de werktijden.

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- *"Haru ni Sakura" - - "Cherry Blossoms in the Spring" -*
"... in the peaceful days, where the cherry blossoms are so beautiful in the spring..."

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Identification of salt-induced genes of *Zygosaccharomyces rouxii* by using *Saccharomyces cerevisiae* GeneFilters®. S.A. Schoondermark-Stolk, E.G. ter Schure, C.T. Verrips, A.J. Verkleij, J. Boonstra (2002). FEMS yeast research, 2, 525-532.

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Rapid identification of target genes for 1-methyl-3-butanol production in *Saccharomyces cerevisiae* S.A. Schoondermark-Stolk, M. Jansen, J.H. Veurink, A..J. Verkleij, C.T. Verrips, G.J.W. Euverink, J. Boonstra, L. Dijkhuizen (2005) Submitted.

Identification of genes that are expressed similar to *HXT5* upon an increase in G1 phase duration. R. Verwaal, V.J. Winter, S.A. Schoondermark-Stolk, A.J. Verkleij, C.T. Verrips and J. Boonstra. Manuscript in preparation.